NUCLEAR DIVISION IN NINE SPECIES OF SMALL FREE-LIVING AMOEBAE AND ITS BEARING ON THE CLASSIFICATION OF THE ORDER AMOEBIDA

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(*Communicated by T. Goodey, F.R.S.—Received 30 October* 1951)

CONTENTS

A study of nine species of small free-living amoebae has been made under standardized and reproducible cultural conditions, by a new method that enables specimens in all stages of division to be obtained easily. In all species the resting nucleus shows a Feulgen-negative nucleolus and Feulgen-positive chromatin granules. Nuclear division in these species and in other amoebae described by other workers is of two main types on which it is proposed to create two new families—Schizopyrenidae and Hartmannellidae. In Schizopyrenidae, the type genus *Schizopyrenus* n.g. and two other genera, *Naegleria* and *Didascalus* n.g., are defined. *Naegleria gruberi*, *Didascalus thorntoni* n.sp., *Schizopyrenus russelli* n.sp., *S. erythaenusa* n.sp. and *S. atopus* n.sp. are described. In Hartmannellidae the type genus *Hartmannella* is defined. *H. glebae*, *H. rhysodes* n.sp., *H. leptocnemus* n.sp and *H. agricola* are described. The relation of the proposed classification to previously defined families and genera of amoebae, and its bearing on phylogeny are discussed.

I. Introduction

Wenyon (1926) divided the order Amoebida Calkins into four families: (1) Family Amoebidae Bronn—amoebae that are not able to form flagella. (2) Family Paramoebidae Poche—amoebae which, in addition to a nucleus of the usual type, possess an accessory body (Nebenkörper) which, during division, divides with the nucleus. (3) Family Dimastigamoebidae Wenyon—amoebae which, in adult form, are able under certain conditions to form two or more flagella, by means of which they progress as flagellates. (4) Family Rhizomastigidae Calkins—amoebae which are provided with a single flagellum during the greater part of the free-living existence.

Unless a definite connecting link is established between the amoebae having temporary flagella under certain physiological conditions and the organisms belonging to the family Rhizomastigidae it is wiser to place the latter in Mastigophora as has been done by Calkins (1933), Kudo (1946) and others. The organisms included in the remaining families by Wenyon (1926) have been accepted by protozoologists to belong to amoebae. The system of classification, however, adopted by the various authorities differs in details. Calkins (1933) divided amoebae into four families: (1) Family Bistadiidae Doflein—to include amoebae characterized by two interchangeable phases, amoeboid and flagellate. (2) Family Amoebidae (authors generally em. Doflein, em. Calkins)—the free-living amoebae are placed in this family. (3) Family Endamoebidae Calkins—to include exclusively the parasitic forms. (4) Family Paramoebidae Poche. Kudo's (1946) classification is based on Wenyon (1926) and Calkins (1933). According to him the amoebae should be divided as follows: (1) Family Dimastigamoebidae Wenyon. (2) Family Amoebidae Bronn—to include free-living and a few parasitic amoebae. (3) Family Endamoebidae Calkins. (4) Family Paramoebidae Poche.

It is not intended to review the literature on the classification of amoebae into families but to show that, so far, no system of classification has been put forward which is based on probable phylogenetic relationships of amoebae.

It appears to the writer that the creation of families, based on such characters as the temporary production of flagella by some amoebae under physiological conditions not properly understood, their parasitic or free-living nature and the possession of an accessory body 'Nebenkörper' is unsound. It does not bear any relation to the probable evolution of the organisms included in the order Amoebida. It is very well known that *Entamoeba histolytica* and other intestinal amoebae can be grown in culture indefinitely on suitable bacterial food like most of the free-living non-parasitic forms. The mere fact that some amoebae become parasitic does not justify the creation of the family Endamoebidae. The production of temporary flagella under conditions imperfectly understood is equally unsuitable as a character for the creation of a family. In some amoebae it is neither easy to produce temporary flagella nor is this character as consistent as was assumed by those who created the families Dimastigamoebidae or Bistadiidae. The question of the production of flagella will be discussed in connexion with the amoebae described in this work. Similarly, the possession of 'Nebenkörper' by amoebae has not much significance when attempts are made to trace the evolutionary history of the order Amoebida.

The genera and species among small free-living, intestinal and parasitic amoebae have been created by the earlier workers largely on the basis of their nuclear structure and their mode of nuclear division. A number of memoirs dealing with the nuclear division in small free-living amoebae have appeared since the work of Vahlkampf (1905). A careful study of these memoirs clearly shows that conflicting views have been put forward by the different workers who carried out cytological investigations. The confusion that prevails can be mainly assigned to two principal causes: first, the study of amoebae grown in nutrient media with uncontrolled bacterial food which may lead to the production of abnormal forms, and secondly, the use of cytological techniques which are not specific for the location of chromatic and non-chromatic materials during all the stages of nuclear division.

The great majority of the earlier workers who studied the nuclear division in small free-living amoebae grew them in rich nutrient liquid media with uncontrolled bacterial food. Two chief methods were used: (1) cover-slips were put at the bottom of the liquid media for the amoebae to grow on them; (2) cover-slips were floated on the surface hoping that amoebae would crawl on them and multiply there. After various intervals the cover-slips were removed and cytological preparations were made. Rich nutrient media, either solid or liquid, are often unsuitable for amoebae, because they may encourage the growth of inedible bacteria and possibly of organisms producing toxic substances (Singh 1941, 1945, 1946, 1947*a*, *b*, 1948*a*). The unsuitability of such methods for getting the stages of nuclear division can be judged by the following remarks of Dobell (1914) who took great pains to study all the stages of nuclear division in free-living amoebae. He says: 'I have studied altogether more than a dozen different species of free-living amoebae. Some of these have been obtained from the infusion of soil, hay and other organic substances; others from fresh water; others again from sea. It is, of course, necessary, in the case of every form to discover at the outset the particular medium which is best suited to it; and discover what it eats, and then take care that it is properly fed' (p. 142). 'I may add here that chance plays a large part in determining the success—or the reverse—which rewards the efforts of those who try to study the method of nuclear division in many species of amoebae. On several occasions I have worked extremely hard for several weeks at a flourishing culture of amoeba—making scores of preparations systematically at all times of the day, and examining thousands upon thousands of individuals. On at least two such occasions I have been compelled to cease from the investigation from sheer exhaustion—without having found a single dividing organism. On one occasion, I found an almost complete series of stages in division in the first preparation that I made from a culture of amoeba—and in large numbers of subsequent preparations of the same forms not a single stage' (p. 146). Dobell was successful in getting the details of the nuclear division in only two species of amoebae during a period of seven years.

The use of haematoxylin and of other non-specific aniline dyes and their combinations as the main criteria for differentiating the nuclear structure and nuclear behaviour during division has created great confusion in the past. Extraordinary differences in the behaviour of the constituents of the nucleus during division have been claimed. The structure which has been called the karyosome, but which should be called nucleolus because it is Feulgen-negative,* has been supposed to contribute either partly or completely to chromosomal chromatin in some forms and not in others. The 'peripheral chromatin' has been found to be completely absent in some amoebae and present in others. Claims have been made

* Minchin (1912) in describing the nuclear structure in Protozoa says: 'In addition to the achromatic framework, plastin is commonly, if not invariably, present in the form of masses or bodies which receive different names, according as they consist of pure plastin or of plastin impregnated to a greater or less extent with chromatin. In the vesicular type of nucleus, the endosome may perhaps consist, in some cases, of pure chromatin, but in most cases, if not always, it is composed of a matrix or ground-substance of plastin in which the chromatin is lodged. An endosome of this kind is termed a *Karyosome*, or chromatin-nucleolus; as a rule it has the form of a rounded mass, occupying the centre of the nucleus, sometimes of more than one such mass, but in a few cases it may have the form of a crescent or cap ("calotte") closely applied to the nuclear membrane. In the granular type of nucleus, on the other hand, there may be one or more masses of pure plastin containing no chromatin; such a body is termed a *nucleolus* simply, or a "plastin-nucleolus"' (p. 76).

that 'peripheral chromatin' sometimes gives rise to chromosomes and sometimes disappears completely during division of the nucleus. It may be emphasized that before the Feulgen technique came into use, it was impossible to locate chromosomal chromatin through the various phases of nuclear division. It is now clear that the structure of the resting nucleus in amoebae is of similar pattern to the nucleus of higher plants and animals.

The difficulty of getting the various normal stages of nuclear division and the use of unsuitable cytological techniques resulted in the creation of a large number of species of small free-living amoebae, the great majority of which can be identified only by those who described them. A satisfactory classification of amoebae remains to be found.

It was thought that the study of the nuclear division and other characters in small free-living amoebae, under controlled and reproducible cultural conditions combined with the use of Feulgen reaction to distinguish between chromatic and non-chromatic substances, might be of help in classifying small free-living amoebae. An opportunity of isolating a large number of strains of amoebae was found in connexion with work on the effects of artificial fertilizers and dung on the numbers of amoebae in Rothamsted soils (Singh 1949). A culture method, based on the selective feeding of amoebae on varied bacterial strains, for the study of the nuclear division in small free-living amoebae was developed (Singh 1950). This method enables one easily to get all the stages of normal nuclear division whenever desired without relying on chance. The use of Feulgen reaction has shown that in all the thirteen strains of amoebae, consisting of at least nine distinct species, the resting nucleus contains Feulgen-positive granules which give rise to chromosomes during nuclear division. The nucleolus neither contains Feulgen-positive chromatin nor does it give rise to chromatin at any stage in nuclear division. This brings the mode of nuclear division in the small free-living amoebae into line with that found in higher animals and plants. The nuclear division in these amoebae is neither of such a primitive nature nor is there such diversity of type as one is led to believe from much of the earlier literature, and according to their mode of nuclear division the amoebae fall into two main groups.

II. Material and methods

During 1945 to 1948 a large number of strains of small soil amoebae were isolated in 'pure mixed' cultures. Whenever the amoebae developing on the culture plates, used in the quantitative estimation of Protozoa (Singh 1949), appeared different either in their morphological characters or in the characters of their cysts, they were isolated in pure-line cultures. The single cysts or single trophic forms were grown on non-nutrient agar (1·5% agar in 0·5% sodium chloride; pH 6·6 to 7·0) plates supplied with a young culture (2 to 5 days old) of *Aerobacter* sp. grown on nutrient agar slopes (Strain 1912; Singh 1941). This bacterium has been found to be one of the best sources of food for soil amoebae and other groups of soil micro-predators during an extensive study of the selection of very varied bacterial food by several groups of soil holozoic organisms (Singh 1941, 1945, 1946, 1947*a*, *b*, 1948*a*, *b*). To make sure that the cultures of amoebae were pure line, the amoebae were grown once again from single cysts by the method described above. The single cysts were isolated by micro-pipettes in drops of sterile water and were washed several times in water before being transferred to the non-nutrient agar plates supplied with *Aerobacter* sp. as food. All precautions were taken to work under sterile conditions.

No detailed record of the occurrence of the various species of amoebae in the differently manured soils of Barnfield and Broadbalk at Rothamsted has been kept. The soils from which they were isolated and the soil dilutions in which they appeared will be given in connexion with the work dealing with the nuclear division and other characters of the amoebae.

In the early stages of the work on nuclear division cover-slips or slides were put in Petri dishes with enough sterile tap or distilled water to cover them. Mass inoculations of amoebae and bacteria were made, and after various intervals the slides and cover-slips were removed and cytological preparations were made in the usual way. By this method some of the division stages in two or three species of amoebae were obtained after examining very large numbers of amoebae. In later experiments dilute nutrient media, such as hay infusion, nutrient broth and soil extract, were tried in place of distilled or tap water without much success. During a period of 4 to 5 months a very large number of cytological preparations at various intervals in the day and at night were made, but these methods did not produce much success. The amoebae growing in liquid media remained active, and some of them divided during a period of 12 to 40 h and then they either encysted or died. In a few species of amoebae large numbers of active forms were observed over a period of 2 to 3 days without finding many dividing individuals. It was realized that a suitable culture method would have to be developed for easily getting the various stages of nuclear division. Such a method is briefly described below.

The amoebae that were required for cytological preparations were subcultured every 24 to 48 h on non-nutrient agar plates, in order to get healthy and actively dividing forms. They were then grown on thin films of non-nutrient agar on slides. The amoebae were then fixed by the usual cytological methods and the slides were brought to water. At this stage the film of agar was removed by giving the slide a gentle shake, leaving the majority of amoebae stuck on the slide. By this method a large number of normal, young and actively dividing amoebae can be found on a single slide. As they are confined to a small area it is easy to find dividing individuals in various stages under an oil-immersion lens. For the details of this culture method for growing small free-living amoebae for the study of their nuclear division see Singh (1950).

Bouin, Schaudinn and Carnoy fixatives were tried in the beginning of the work. As Carnoy fixative (glacial acetic acid 1 part, absolute alcohol 6 parts and chloroform 3 parts) gave very good results, it was extensively used. The amoebae were fixed for 30 to 50 min and then they were put into 90% ethanol for 24 h. They were then brought into 70, 50 and 30% ethanol successively, leaving them in each for several minutes and then into distilled water. After the removal of the agar film, the amoebae stuck to the glass slide were stained either by iron-alum haematoxylin or by the Feulgen reaction.

In the beginning of this work iron-alum haematoxylin was chiefly used. Slides, after the removal of the film of agar, were placed in 2% iron alum in water for 6 to 7 h, washed in distilled water and transferred to 0·5% haematoxylin in water. They were left overnight in the stain, and were differentiated in 1% iron alum, washed under running tap water for $\frac{1}{2}$ h, dehydrated and mounted in the usual way in balsam. Although excellent preparations were made by this method, it was impossible to trace the chromosomal chromatin, with any confidence, through the various stages of nuclear division.

Both the chromatic and non-chromatic materials take the haematoxylin stain, the latter staining more deeply. In order to distinguish between chromatic and non-chromatic substances Feulgen reaction was tried. After a little experimentation in the time of hydrolysis and staining in the leuco-basic fuchsin Feulgen reaction gave very good results. It has been found most valuable in clearing the controversial points regarding the origin and behaviour of chromosomal chromatin through the various stages of nuclear division in amoebae. A very brief description of the Feulgen-staining technique is given below in the hope that it may be of use to workers studying the nuclear cytology of the free-living and parasitic amoebae.

The Carnoy-fixed amoebae, after the removal of the agar film, were hydrolyzed in N-HCl at 60° C for only 4 to 5 min. This time of hydrolysis was found most satisfactory for the subsequent staining of the nuclear chromatin of all the species of amoebae studied. The amoebae were then stained in leuco-basic fuchsin for 3 to 4 h by the method described by De Tomasi (1936). The counter-staining with light green, as described by Semmens & Bhaduri (1939), was found to be very helpful in studying the location and the behaviour of non-chromatic material in the resting and the dividing nuclei.

It is well known that the size of amoebae is influenced greatly by the cultural conditions and the food supply. In this work standardized and reproducible cultural conditions have been used. All the species were grown on non-nutrient agar plates supplied with *Aerobacter* sp. (Strain 1912) as food. The active forms and cysts were examined in the living condition under an oil-immersion lens by putting a no. 1 cover-slip on the culture of amoebae on the agar.

A comparison of the Feulgen preparations with those stained with iron-alum haematoxylin shows how difficult it is in the latter preparation to distinguish between chromatic and non-chromatic materials in the dividing nucleus at certain stages. The results obtained with iron-alum haematoxylin have been included in this work to illustrate the confusion that its use has created in the past.

III. Nuclear division and other characters in small free-living amoebae

(1) *Naegleria gruberi* (Schardinger)

This is a common free-living amoeba studied by several workers and recorded from various soils by Sandon (1927) and others. The description given here is based on three strains isolated by Dr E. G. Pringsheim (Botany School, University of Cambridge) from fresh water and kindly sent to me. The study of the nuclear division in this amoeba has created more confusion, in the past, than that of perhaps any other form among the small free-living amoebae. Recently, Rafalko (1947) has given a good account of the nuclear division using Feulgen reaction. My observations are largely in agreement with his findings.

Morphology, etc.

The amoebae are variable in size and shape. In a rounded condition they have a diameter of about 15 to 30μ. They are very active in young culture and very often assume a *limax*-like form and move in the characteristic slug-like manner. There is a well-defined

ectoplasm and endoplasm (figure 1 *a*, *b*). A single contractile vacuole is present in each amoeba.

The living cysts are round or slightly oval in outline and very variable in size. Each cyst contains a single nucleus. The nucleolus can be clearly seen, although no chromatic granules could be distinguished in unstained cysts. The cysts usually show an irregular outer layer, and the majority of them appear to be pierced by two or sometimes more pores (figure 1 *c*, *d* and *e*). These pores seem to be plugged with some kind of structureless substance.

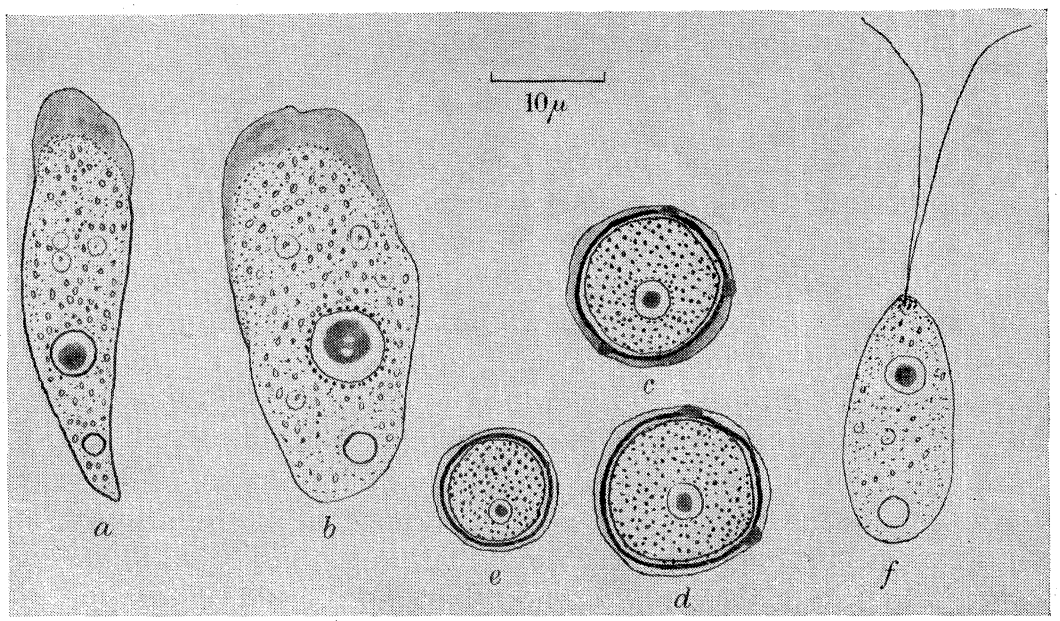


FIGURE 1. *Naegleria gruberi*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, *e*, cysts; *f*, flagellate stage.

The flagellate stage can be easily produced in *Naegleria gruberi*. When mass inoculations of the actively growing amoebae from non-nutrient agar plates are made into hanging-drop preparations in sterile-distilled or tap water, a very large percentage of them assume the flagellate stage within a period of 6 to 24 h between 20 and 21° C. The flagellates have a more or less rigid oval shape and move actively in water. Each individual possesses two flagella, slightly longer than the body, arising from the anterior end (figure 1*f*). There is a single large contractile vacuole situated at the posterior end. No detailed study of the transformation of the amoebae into the flagellates and vice versa has been made. Judging from the earlier literature on the production of the flagellate stage by amoebae, it appears that *N. gruberi* has been primarily responsible for the creation of the family Dimastigamoebidae or Bistadiidae by the various authors. It will be shown later that some amoebae do not readily produce flagella and that this character is not suitable for the creation of a family.

Structure of the resting nucleus in fresh and in stained preparations

The resting nucleus, in the living condition, consists of a distinct central spherical nucleolus surrounded by a clear zone. Fine radiating threads and chromatin granules could not be distinctly seen. In Feulgen preparations, Feulgen-negative nucleolus and Feulgen-

positive, faintly red-staining chromatin granules, situated near the nuclear membrane, can be clearly seen (figure 22). The nucleolus stains deeply with the light green counter stain. Occasionally one or two unstained patches can be seen inside the nucleolus (figures 2, 22). In some nuclei the chromatic granules seem to be arranged in the form of threads (figure 22) as figured by Rafalko (1947). In iron-alum haematoxylin preparations thread-like structures radiating from the nucleolus are clear in some nuclei (figure 2). The nucleolus stains more deeply than the chromatic granules and resists decolorization with 1% iron alum much longer than the chromatic substance. Occasionally the chromatin is seen in patches (figure 2). Usually one nucleus is found in each amoeba, though two or more nuclei can often be seen in an individual.

Mitotic division

Prophase. The amoebae do not become rounded or motionless during division. The beginning of the nuclear division is marked by the swelling of the nucleus and the elongation of the nucleolus (figures 3, 23). The chromatic granules lie beside the nucleolus (figures 3, 4, 23, 24) and are clearly seen in Feulgen preparations. These granules begin to fuse, and occasionally there appears to be a solid ring of chromatic material surrounding the nucleolus (figure 25). The nucleolus later on assumes usually a dumb-bell-shaped appearance and divides into two halves known as 'polar masses' (figures 4, 5, 26). Sometimes the 'polar masses' seem to be connected by a thread-like structure (figure 5).

Metaphase. After the formation of the 'polar masses', a solid mass of Feulgen-positive chromatin is seen occupying the position of the equatorial plate (figures 6, 26). No individual chromosomes could be made out at this stage. The spindle connecting the 'polar masses' can be seen, though no distinct spindle fibres could be distinguished in the great majority of the amoebae. No division of the chromosomes could be seen.

Anaphase. It appears that the band of chromatic material divides into two and each half moves towards its pole (figures 7, 8, 27 to 30). These stages are very distinct in Feulgen preparations. In iron-alum haematoxylin-stained nuclei occasionally one gets figures where the presence of chromosomes could not be ascertained (figure 9). Certain granules lying on the spindle give the false appearance of chromosomes. Judging from Feulgen preparations these granules are not chromatic in nature. The chromatic material at this stage lies in contact with the 'polar masses'.

After the Feulgen-positive chromatic material has moved to the two poles, certain granular non-chromatic substance, which Rafalko (1947) has called the 'interzonal body', can be distinctly seen (figures 10, 30) lying half-way between the two 'polar masses'.

All the figures in this paper have been drawn from fixed and stained preparations at the same magnification under an oil immersion lens.

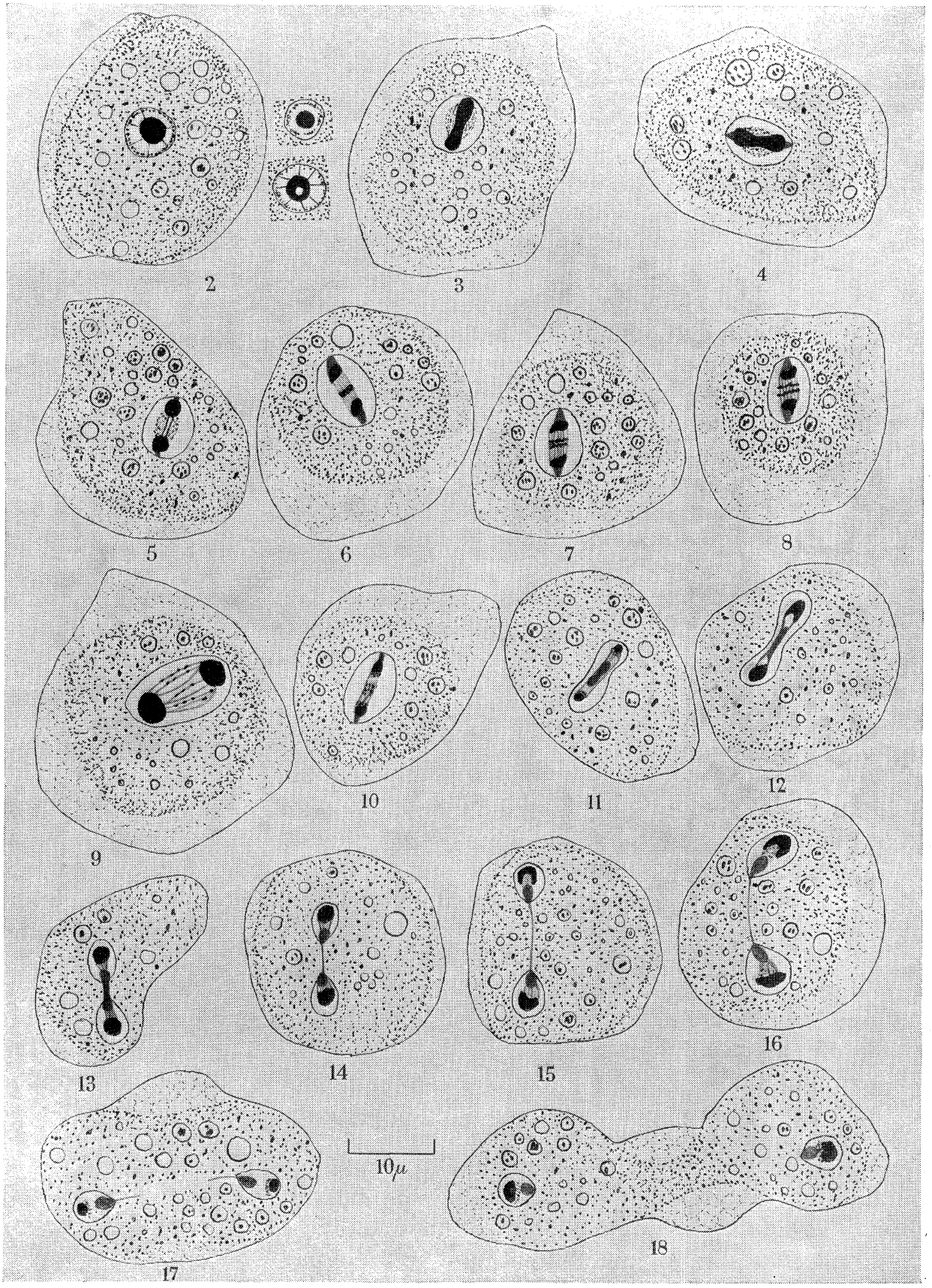
Naegleria gruberi. FIGURES 2 to 18. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 2. Ordinary individual and the structure of three resting nuclei.

FIGURES 3 to 18. Successive stages in division.

FIGURES 4 to 8 and 10. Showing the presence of 'polar caps'.

FIGURES 10 to 18. Showing the formation of 'interzonal body' and its division into two equal halves.



FIGURES 2 to 18

The origin of these granules could not be determined, but probably they arise from the nucleolus during its division into 'polar masses'. Rafalko (1947) has given a convincing and good account of the 'interzonal body' from the time it is first seen and its behaviour during the subsequent stages of the nuclear division. My observations are in accord with his findings. The 'interzonal body' increases in size and divides into two (figures 11, 13). The nuclear membrane persists throughout division. It becomes elongated and constricts, giving rise to two daughter nuclei (figures 11, 12, 13 and others).

Telophase. After the nucleus has divided into two, the two 'interzonal bodies' are still connected for a time by a thread-like structure (figures 14 to 17, 32, 33). The amoeba later on becomes elongated and constricts in the middle to give rise to two daughter individuals. The 'interzonal body' and the 'polar mass' of the daughter nuclei fuse together to give rise to the nucleolus; the chromatic material is broken up into granules, and these granules occupy the position as seen in the resting nuclei (figures 16 to 21, 33 to 37). In iron-alum haematoxylin preparations the chromatic material (figures 12 to 18) is either not distinguishable or only very faintly marked. In Feulgen preparations of the similar stages (figures 33 to 37) both the chromatic and non-chromatic substances can easily be distinguished without any confusion.

Polar caps

Ford (1914) described achromatic caps situated during some of the stages of nuclear division between the ends of the elongated karyosome and the nuclear membrane in a free-living *limax* amoeba. He thought that his amoeba was probably *Amoeba tachypodia* Gläser. Judging by the presence of 'interzonal bodies' as figured by Ford (1914) and Gläser (1912*a*) and of the flagellate stage described by Pietschmann (1929) in *A. tachypodia* it seems certain that this amoeba is a species of the genus *Naegleria*. The 'polar caps', according to Ford, were not clearly seen in iron-alum haematoxylin preparations, but were very distinct when stained by Dobell's (1914) alcoholic iron-haematin method. No difficulty was encountered in finding Ford's 'polar caps' in *N. gruberi* by Rafalko (1947) and the writer either in iron-alum haematoxylin or Feulgen preparations counterstained with light green (figures 4 to 8, 10, 25, 27, 29). In iron-alum haematoxylin preparations they are very clearly seen situated above the 'polar masses' (figures 6 to 8 and others). How 'polar caps' arise and what role they play in the nuclear division could not be ascertained.

No centrioles at the pointed ends of the 'polar caps' as described by Rafalko (1947) and others could be seen. The writer is of the opinion that no centrioles are present at any stages during the nuclear division in *N. gruberi*.

Critical remarks

The nuclear structure and the mode of nuclear division in amoebae which have 'polar masses' and 'interzonal bodies' have been studied in the past by haematoxylin and other non-specific chromatin stains. This has led to great confusion in interpreting the results obtained by the different workers. It is not intended here to review the literature but to show that in these amoebae there is uniformity and not such a great diversity in the nuclear structure and the nuclear division as was claimed in the past.

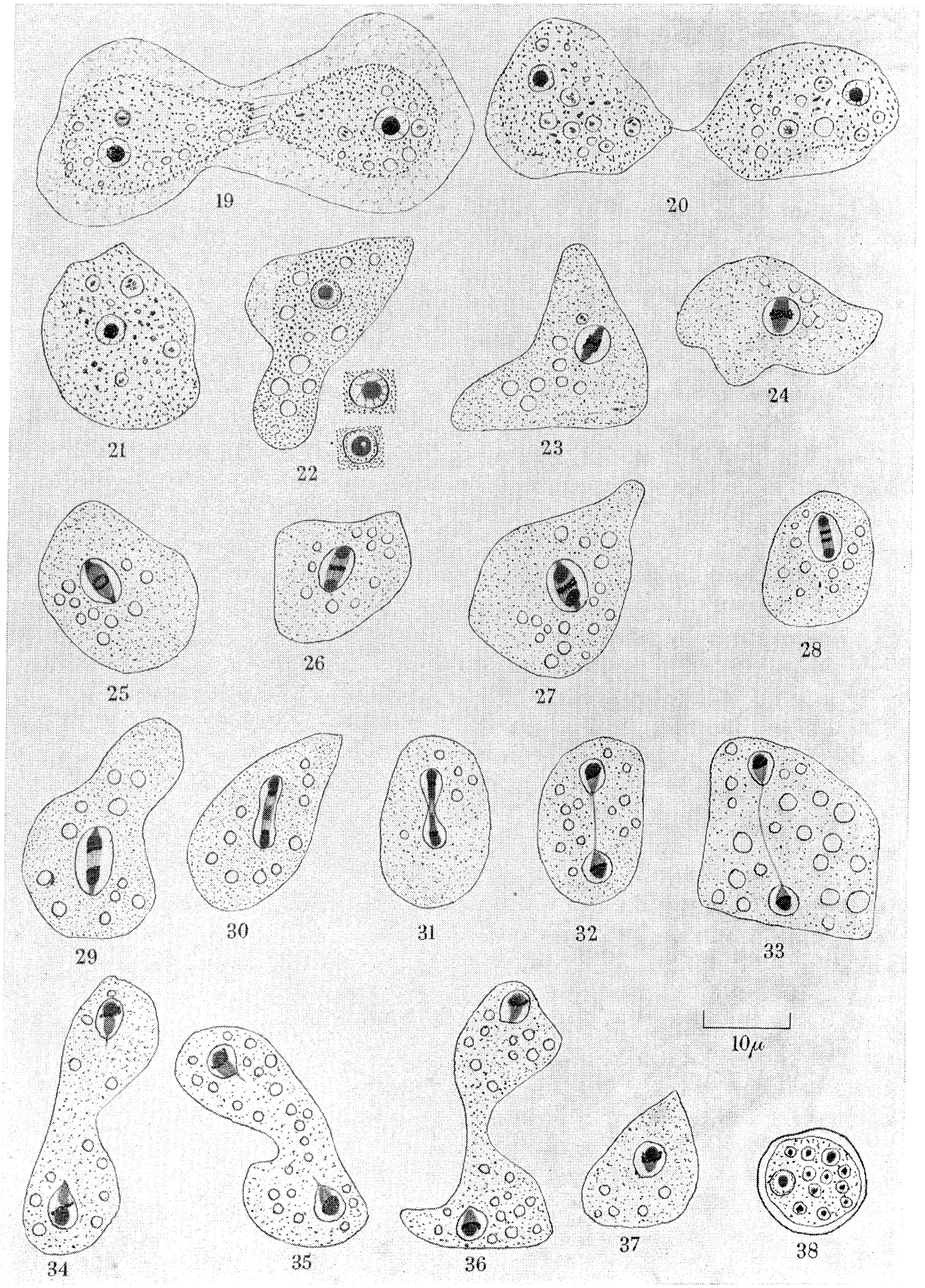
Vahlkampf (1905) was the first to discover the presence of 'polar masses' during division in a *limax* amoeba, although according to him the nucleolus (karyosome) contained both the chromatic and the non-chromatic substances. From his figures 13, 18 to 21, plate 6, it seems certain that in his amoeba 'interzonal bodies' as described by the writer and Rafalko (1947) were also present. The credit for having first observed or figured these structures must therefore be assigned to Vahlkampf (1905).

Although the origin of chromosomes has been attributed to the nucleolus (karyosome), to the 'peripheral chromatin' or to both, some workers, like Chatton (1910*b*), Wasielewski & Herschfeld (1910), Wasielewski & Kühn (1914), Jollos (1917) and others, thought that chromosomes arose from the chromatin outside the nucleolus. The use of Feulgen reaction has shown that in *N. gruberi* the nucleolus neither contains chromatin nor does it give rise to chromatin at any stage during nuclear division. The chromosomes arise from pre-existing Feulgen-positive chromatin granules present outside the nucleolus.

The 'interzonal bodies' in the early stages of their formation have been taken to be rod-shaped chromosomes by some workers who relied on iron-alum haematoxylin preparations. The observations of Kühn (1920) in *Vahlkampfia bistadialis* and Ivanić (1936) in *V. danubiensis* and others on the presence of well-defined chromosomes need confirmation by the use of the Feulgen reaction before they can be accepted. The work of Zulueta (1917) in *Wasielewskia gruberi* on 'promitosis' and 'sindierésis' found in the same species of amoeba and of Aragão (1909) on *Amoeba diplomitotica* showing two types of nuclear division cannot be accepted unless these amoebae are studied again under suitable cultural conditions and by better cytological techniques.

Wilson (1916) described exogenous and endogenous budding in *Naegleria gruberi*. Such phenomena could not be observed in the same species of amoeba investigated by Rafalko (1947) and the writer using Feulgen technique. It is very doubtful if in any small free-living amoeba such budding can be seen in normal healthy forms. The extrusion of chromatin from the nucleus into the cytoplasm as described by Wilson (1916) could not be confirmed by the use of Feulgen reaction. The occurrence of this phenomenon in amoebae needs confirmation by reliable cytological techniques before it can be accepted.

Judging by the earlier literature it is not possible to say how many valid species of amoebae exist in the genus *Naegleria*. It may be that the majority of the previously described species that have 'polar masses', 'interzonal bodies' and temporary flagellate stages are *N. gruberi* (Schardinger). Schardinger (1899) first described this species in a diarrhoeic stool, as having amoeboid and flagellate stages. It has been put into several genera, such as *Amoeba*, *Dimastigamoeba*, *Vahlkampfia* and *Wasielewskia*. In order to create valid species in this genus, it is necessary that the morphological and other characters of amoebae having 'polar masses', 'interzonal bodies' and temporary flagellate stages should be studied under standardized and reproducible cultural conditions. Unless this is done, it will not be possible to create species of *Naegleria* which can be identified with ease by other workers. By the study of the nuclear structure and the mode of nuclear division, one can only be certain of the genus and not of the species.



FIGURES 19 to 38

(2) *Didascalus** n.g.

The new genus is defined later on p. 454.

Type species *Didascalus thorntoni*† n.sp.

Miss L. M. Crump isolated this organism from Broadbalk field farmyard manured (plot 2) at Rothamsted several years ago, and she kindly gave me a culture of it. Recently she has given a brief description of this amoeba (species Z) in her work on the influence of bacterial environment on the excystment of amoebae from soil (Crump 1950). It resembles *Vahlkampfia soli* described by Martin & Lewin (1914) in several respects. Its similarities to and differences from *V. soli* will be discussed after recording my own observations.

Morphology, etc.

The amoebae are variable in shape and size. In a rounded condition they are approximately 15 to 20μ across. In young cultures they are very active and often move in the characteristic *limax* form. During movement the ectoplasm and endoplasm are very distinct (figure 39*a*,*b*). There is a single contractile vacuole. Each amoeba usually contains a single nucleus, although four or six nuclei can often be seen in a single individual. The animals possessing more than one nucleus appear quite normal but are larger than the uninucleate forms.

The living cysts are characteristic and very variable in size. They are round having a single wall. The outside of the wall consists of a fairly thick transparent gelatinous layer (figure 39*c*, *d* and *e*). The cysts stain deeply with aniline blue acetic stain, the gelatinous layer remaining colourless. The nucleolus inside the nucleus can be seen in some of the living cysts, although no chromatin granules can be distinguished.

* This genus is named from the Greek word Didascalus (διδάσκαλος) meaning schoolmaster—one who is prone to the whip.

† The specific name is given in honour of Dr H. G. Thornton, F.R.S., in whose department I have had the privilege of working for many years.

Naegleria gruberi. FIGURES 19 to 21. Fixed in Carnoy and stained with iron-alum haematoxylin and FIGURES 22 to 38 fixed in Carnoy and stained with Feulgen reaction and light green

FIGURES 19 and 20. Successive stages in division.

FIGURE 21. An amoeba just after division.

FIGURE 22. Ordinary individual and three resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 23 to 36. Successive stages in division showing the behaviour of chromatin and the nucleolus.

FIGURES 25, 27 and 29. Showing 'polar caps'.

FIGURE 30. Showing the non-chromatic 'interzonal body' after the chromosomes have moved to the poles and are lying in contact with the 'polar masses'.

FIGURES 31 to 36. Showing the 'interzonal bodies'.

FIGURE 37. Showing the fusion of the 'interzonal body' and the 'polar mass' to give rise to the nucleolus in an amoeba just divided.

FIGURE 38. A cyst showing Feulgen-negative nucleolus and Feulgen-positive chromatin granules in the nucleus.

The flagellate stage in *Didascalus thorntoni* could not be produced as readily as in the case of *Naegleria gruberi*. A few flagellates were first observed by the writer in drops of water that had condensed on the agar surface used for growing amoebae. When young and actively growing amoebae are subcultured, a few flagellate forms can be seen after 20 to 48 h at 20 to 21° C by the above method. By mass inoculation of the actively growing forms in hanging-drop preparations in sterile distilled or tap water a few flagellates out of a few thousand amoebae appear during a period of 20 to 40 h at 20 to 21° C. One cannot be sure of finding the flagellates in all the hanging-drop preparations. The flagellates usually have a more or less rigid oval shape and swim actively in water. Each individual possesses two flagella, longer than the length of the animal, arising from the anterior end. In specimens stained with aniline blue acetic a rhizoplast connecting the basal granules to the nucleus can be seen (figure 68). No detailed observations of the transformation of the amoebae into flagellates and vice versa have been made.

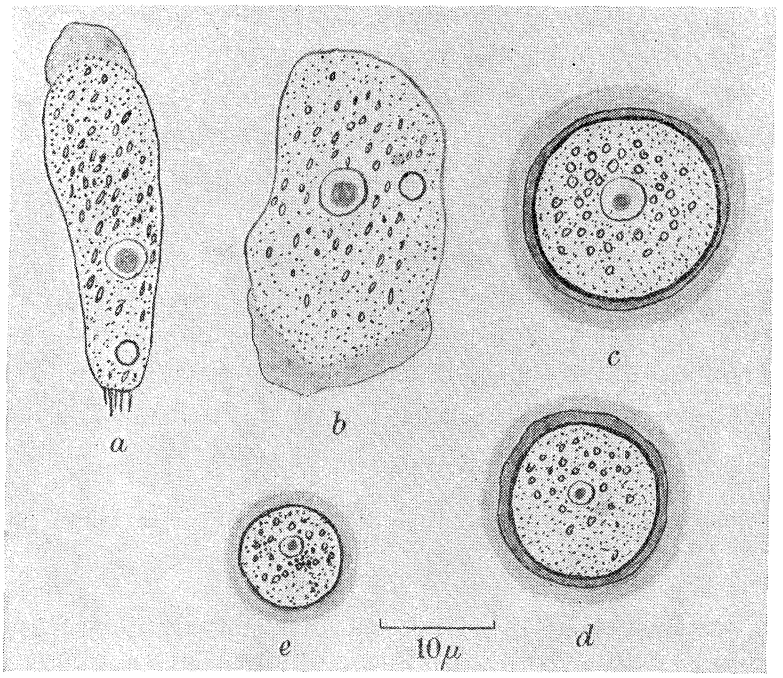


FIGURE 39. *Didascalus thorntoni*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, *e*, cysts.

Structure of the resting nucleus in fresh and in stained preparations

The resting nucleus, in the living condition, consists of a central spherical nucleolus surrounded by a clear space. No chromatic granules or radiating threads could be seen. During movement the nucleolus often assumes an elongated shape and then returns to its spherical form. This transformation of the shape of the nucleolus suggests that it consists of an elastic substance. In Feulgen preparations there is a large Feulgen-negative nucleolus and Feulgen-positive faintly red-stained chromatic granules (figure 73). These granules lie near the nuclear membrane. Non-stainable patches in the nucleolus can be seen in preparations counter-stained with light green (figure 73). In iron-alum haematoxylin preparations the nucleolus stains deeply, and thread-like structures radiating from it can be seen in some nuclei (figure 40).

Mitotic division

Prophase. The amoebae do not become rounded during division. The nuclear division is initiated by the nucleolus becoming elongated. The chromatic granules move from their position near the nuclear membrane and lie on or near the nucleolus (figures 41, 74, 75). The nucleolus becomes dumb-bell-shaped, and the Feulgen-positive granules begin to fuse together and lie as a band at the centre (figure 76). The dumb-bell-shaped nucleolus divides into two halves known as 'polar masses' (figures 43 to 46, 77). The 'polar masses' seem to be connected for a short time (figures 43 to 45).

Metaphase. After the formation of 'polar masses', a band of deeply staining Feulgen-positive chromatin can be seen occupying the position of the equatorial plate (figure 77). The chromosomes could not be clearly distinguished. A similar stage is shown from an iron-alum haematoxylin preparation in figures 45 and 46. The spindle connecting the 'polar masses' can be clearly seen, although the spindle fibres could not be counted. It appears in some Feulgen preparations that each chromosome divides into two (figure 78). It was found impossible to count the number of chromosomes.

Anaphase. Half the number of chromosomes, after division, move towards one 'polar mass' and the other half towards the other (figures 47 to 50, 79 to 82). Occasionally in iron-alum haematoxylin preparations granules resembling the 'interzonal body', as described in *Naegleria gruberi*, can be seen after the chromosomes come to lie in contact with the 'polar masses' (figure 50). These granules seem to disappear at a later stage, and no formation of definite 'interzonal bodies' could be seen. In figure 51 is shown a thread-like structure connecting the two 'polar masses'. According to some workers this thread is thought to represent the connexion between two centrosomes lying at the two poles of the spindle. The writer could not find any centrosomes; the thread may be a spindle fibre deeply stained in iron-alum haematoxylin.

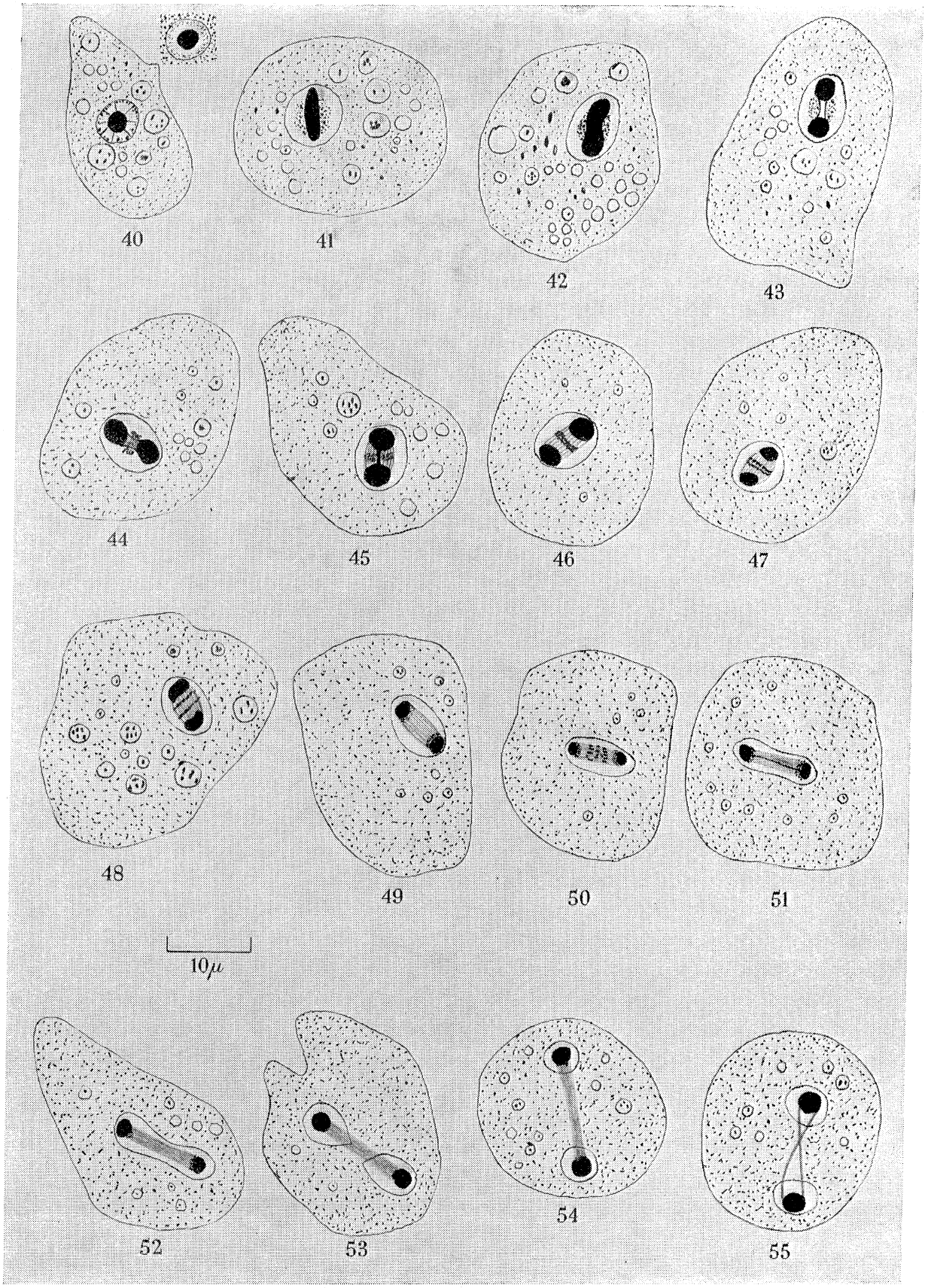
No 'polar caps', as described in *N. gruberi*, could be seen at any stage in the nuclear division.

As in *N. gruberi*, the nuclear membrane persists throughout division. It becomes elongated and constricts into two, giving rise to two daughter nuclei (figures 51 to 53, 82 to 84).

Telophase. After the division of the nucleus, the two 'polar masses' remain connected by the spindle fibres which gradually become narrower and disappear (figures 54 to 58, 84 to 86). The behaviour of the chromatic material can be very easily followed in the early and late telophase in Feulgen preparations. The two bands of chromatic material, after reaching the two 'polar masses' seem gradually to surround the latter (figures 85 to 89). Later on they break up into granules and occupy the position seen in the resting nuclei. The 'polar masses' become nucleoli in the two daughter amoebae. In iron-alum haematoxylin preparations it is impossible to distinguish between chromatic and non-chromatic materials during early and late telophase stages (figures 53 to 60). The amoeba later on becomes elongated and constricts in the middle to give rise to two daughter individuals (figures 58 to 64, 87 to 91), which remain connected by a thin strand of protoplasm for a very short time before they separate (figures 64, 91).

Nuclear division in amoebae having more than one nucleus

Up to eight nuclei have been seen in one amoeba. The multinucleate forms seem to be healthy and the nuclear division takes place normally. When two or more nuclei in



FIGURES 40 to 55

a single individual divide, they all divide at the same time. In figures 69 to 72 are shown some of the division stages of two nuclei present in a single amoeba. It is possible that some of the multinucleate amoeboid organisms in which all the nuclei divide at the same time may have been evolved from an amoeba such as this species.

Critical remarks

At present the only amoeba which can be placed in the genus *Didascalus*, with certainty, is *D. thorntoni* described in this work. Judging by the incomplete and faulty descriptions of the nuclear division given by the earlier workers it is not wise at present to include any other species in this genus.

There are many named species of small free-living amoebae described in the literature that cannot be certainly identified. By abolishing some of these names or assigning them to forms which have been more thoroughly studied, it may be possible, in the long run, to have only species that can be easily identified.

Martin & Lewin (1914) described a new species of amoeba, which they obtained from a sick cucumber soil, and named it *Vahlkampfia soli*. Judging by such characters as the morphology, the possession of an outer gelatinous layer in the cyst and the sporadic production of temporary flagella, it seems possible that Martin & Lewin's organism may be the same as the writer's species of *Didascalus*. From the few figures of the nuclear division given by these authors, it seems certain that their amoeba possessed 'polar masses'. Their figure 16, plate 5, suggests the presence of 'interzonal bodies'. If these structures are accepted in Martin & Lewin's organism, it should be called *Naegleria soli* according to the system of classification adopted in the present work, but as Martin & Lewin do not state if they worked on 'pure-line' cultures obtained from single cells, it is quite possible that they may have had more than one species of amoeba present in their cultures. For the sake of clarity and in order to avoid confusion it seems advisable to erect a new species for the organism described above, and it is accordingly named *Didascalus thorntoni* n.sp. This organism was wrongly named *D. soli* in the writer's article published in *Nature* (Singh 1951).

(3) *Schizopyrenus** n.g.

The new genus is briefly defined on p. 454.

Type species *Schizopyrenus russelli*† n.sp.

This amoeba was isolated on one occasion from Barnfield farmyard manured field (plot 1·0) soil at a dilution of 1/819200 and on the other occasion from Broadbalk un-

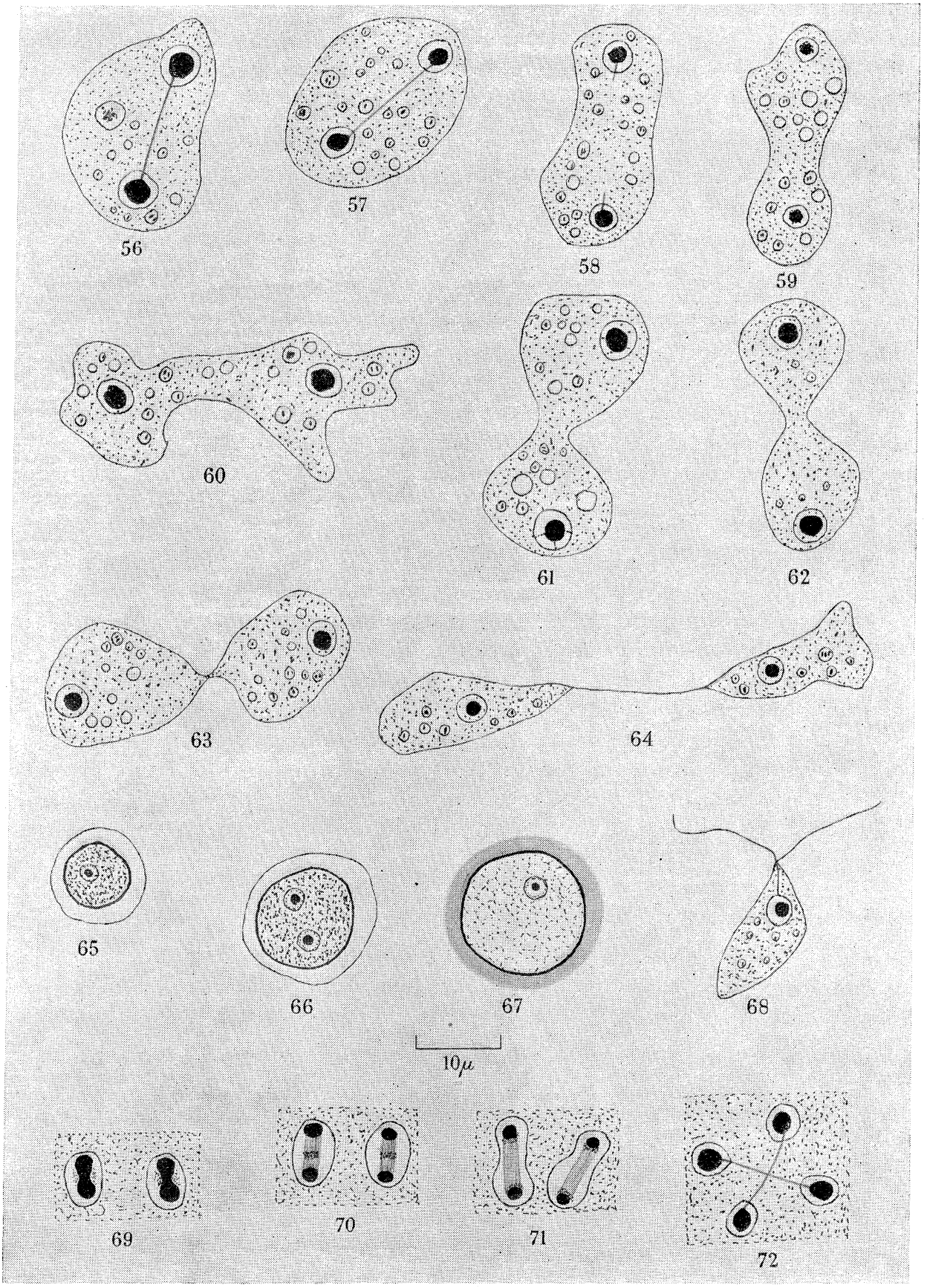
* This genus is named from the Greek σχιζ-, divide; πυρήν, kernel. *Schizopyrenus* from which the family name Schizopyrenidae is derived.

† This species is named after Sir John Russell, F.R.S., late Director of Rothamsted Experimental Station, whose theory of partial sterilization and whose keen interest in soil Protozoa led to the development of soil protozoology.

Didascalus thorntoni or *Schizopyrenus russelli*. FIGURES 40 to 55. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 40. Ordinary individual and the structure of two resting nuclei.

FIGURES 41 to 55. Successive stages in division.



FIGURES 56 to 72

manured soil (plot 3) at a soil dilution of 1/25600. These observations suggest that it may be a common soil species at Rothamsted.

Morphology, etc.

Miss Crump (1950), who used *Didascalus thorntoni* (species Z) and *Schizopyrenus russelli* (species 4) in her work on the influence of bacterial environment on the excystment of amoebae from soil, found that the active forms of these two species were indistinguishable. She says (1950, p. 17): 'Amoeba 4 forms a double-walled cyst and excystment takes place in two stages: first, the inner wall disappears and a small amoeba moves freely within the outer wall; then, in a successful case, the outer wall gives way and the amoeba emerges. If the outer wall remains impenetrable, as may often happen in unfavourable condition, the amoeba dwindles away and ultimately dies. The growth and reproduction in this species is not so fast as it is in species Z.'

Amoebae with more than one nucleus have rarely been found.

The living cysts are spherical in shape and are very variable in size. Each cyst consists of two definite walls (figure 93*c*,*d*). An amoeba which has come out of the inner cyst but still enclosed in the outer cyst wall is shown in figure 93*e*. The nucleolus in the nucleus could be seen only in some cysts and then with difficulty. No chromatic granules could be distinguished. Occasionally in stained preparations a cyst is found with two nuclei (figure 66). Such cysts are much larger than the uninucleate ones.

Repeated efforts to produce a temporary flagellate stage in *Schizopyrenus russelli*, by the methods used successfully with *Naegleria gruberi* and *Didascalus thorntoni*, have completely failed.

Structure of the resting nucleus in fresh and in stained preparations

The resting nucleus in the living and in stained preparations is indistinguishable from the nucleus of *D. thorntoni*. The structure of the resting nucleus shown (figures 40, 73) for *D. thorntoni* holds good for *Schizopyrenus russelli*.

Mitotic division

The amoebae do not become rounded during division. The different stages in nuclear division are indistinguishable from those described for *D. thorntoni* and the figures of the nuclear division given for *D. thorntoni* hold good for *Schizopyrenus russelli*.

Didascalus thorntoni or *Schizopyrenus russelli*

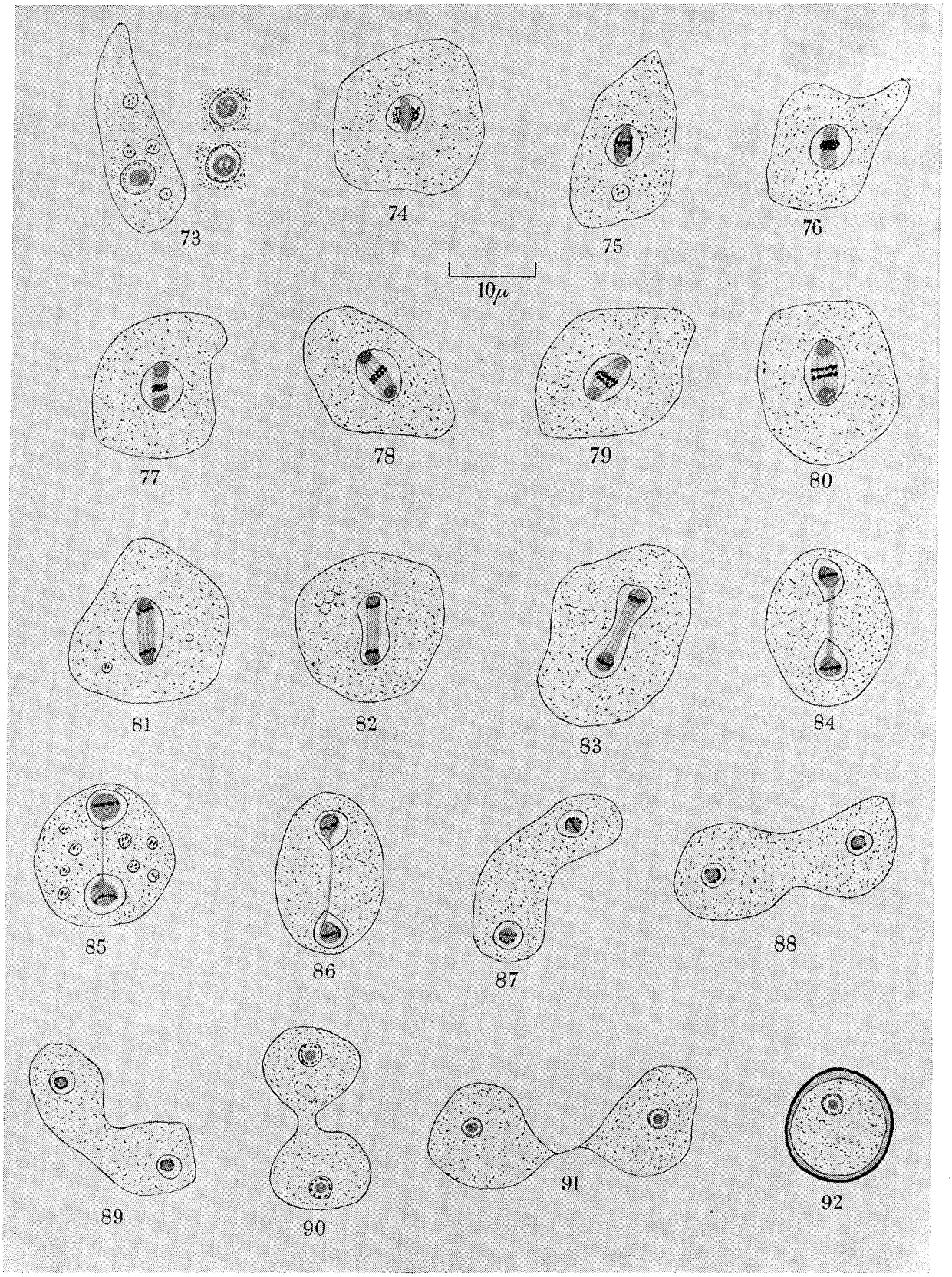
FIGURES 56 to 64. Showing the successive stages in division. Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURES 65 and 66. Cysts of *Schizopyrenus russelli* having one and two nuclei. Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURE 67. Cyst of *Didascalus thorntoni*. Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURE 68. Flagellate stage of *Didascalus thorntoni* stained with phenol aniline blue acetic acid.

FIGURES 69 to 72. Stages of nuclear division in *Didascalus thorntoni* having two nuclei. Fixed in Carnoy and stained with iron-alum haematoxylin.



FIGURES 73 to 92

Critical remarks

S. russelli is a very interesting organism from an evolutionary point of view, because it seems to be closely related to the amoebae which produce a temporary flagellate stage like *Didascalus thorntoni*, and thus may be a connecting link between such amoebae and the more differentiated members of the genus *Schizopyrenus*.

Chen (1937) has described incomplete stages of the nuclear division in an amoeba which he called *Vahlkampfia sonnenscheini* n.sp. His figures show 'polar masses' without the presence of 'interzonal bodies'. Although Chen's description of this amoeba is incomplete, it seems to be a species belonging to the genus *Schizopyrenus*.

(4) *Schizopyrenus erythaenusa** n.sp.

This amoeba was once isolated from Barnfield (plot 4A); i.e. complete minerals + sulphate of ammonia treated soil at a soil dilution of 1/409600 and on another occasion from Broadbalk farmyard soil (plot 2) from a similar dilution. Judging from the presence of this amoeba in very high dilutions of soil, it seems to be common in Rothamsted soils.

Morphology, etc.

The amoebae are variable in size. In a rounded condition they are approximately 15 to 35μ across and are thus slightly bigger than the ones described before. In young cultures they readily produce *limax* forms. There is a distinct ectoplasm and endoplasm the latter usually containing plenty of food vacuoles (figure 94*a*, *b*). A single contractile vacuole is found in each amoeba. Amoebae are usually uninucleate and forms having two nuclei are rare.

Schizopyrenus erythaenusa produces pink pigment when grown on non-nutrient agar with *Aerobacter* sp. as food supply. The description of pigment production given in connexion with *Schizopyrenus atopus*, a species described later on, applies also to *S. erythaenusa*.

The living cysts are very variable in size and are spherical. They possess a single wall consisting of two layers (figure 94*c*, *d*). When an amoeba emerges from the cyst, the wall is not broken down. The nucleolus within the nucleus can be easily seen.

Efforts to produce a temporary flagellate stage have completely failed.

Structure of the resting nucleus in fresh and in stained preparations

In the living condition the nucleus contains a fairly large central nucleolus surrounded by a clear zone (figure 94). No chromatic granules can be seen. In stained preparations

* This species is named from the production of pink pigment in the culture when the amoebae are grown on non-nutrient agar supplied with *Aerobacter* sp. (Strain 1912) as food. (Greek ἐρυθαίνουσα, 'making to blush', from vb. ἐρυθαίνειν.)

Didascalus thorntoni or *Schizopyrenus russelli*. FIGURES 73 to 92. Fixed in Carnoy and stained with Feulgen reaction and light green

FIGURE 73. Ordinary individual and three resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 74 to 91. Successive stages in division showing the behaviour of chromatin and the nucleolus.

FIGURE 92. Cyst of *Schizopyrenus russelli* showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus.

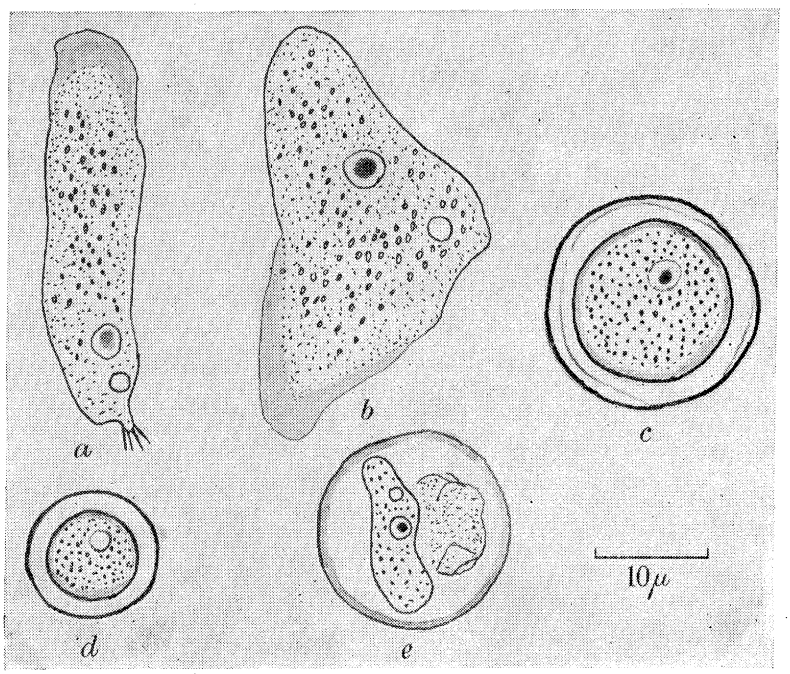


FIGURE 93. *Schizopyrenus russelli*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts; *e*, an amoeba just come out of the inner cyst and still enclosed by the outer cyst wall.

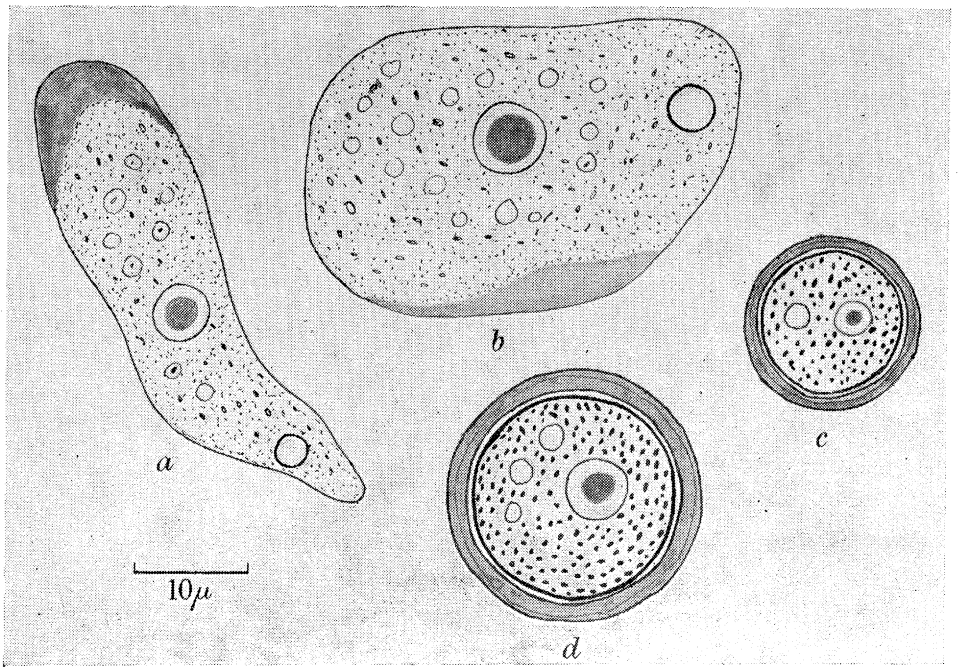


FIGURE 94. *Schizopyrenus erythaenusa*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts.

the structure of the nucleus (figures 95, 111) is similar to that found in *Didascalus thorntoni*. There is a Feulgen-negative nucleolus and Feulgen-positive red-staining chromatin granules (figure 95).

Mitotic division

As the general outline of the nuclear division is similar to that described for *D. thorntoni* only a brief description of the various division stages are given below.

Prophase. The amoebae retain irregular shape during division.

The dumb-bell-shaped nucleolus and the deeply staining chromatin granules in Feulgen preparations are shown in figures 96 to 98. After the formation of 'polar masses' the chromatic granules lie as a band (figures 99, 115, 116).

Metaphase. In some preparations, stained by the Feulgen reaction, the chromosomes lying at the equatorial plate can be easily counted. They are six in number and divide into two halves (figure 100). The spindle with the spindle fibres connecting the two 'polar masses' are clearly seen in some of the iron-alum haematoxylin preparations (figures 117, 119). Occasionally in staining by this method one gets stages as shown in figure 118, where it is impossible to distinguish between chromosomes and other granular structures present on the spindle.

Anaphase. Half the number of chromosomes move towards each of the 'polar masses' as shown clearly in Feulgen preparations (figures 101 to 105). In iron-alum haematoxylin it is impossible to locate the chromosomes during late anaphase and early telophase stages (figures 121 to 123). The nuclear membrane constricts to give rise to two daughter nuclei (figures 101 to 103 and others).

Telophase. After the formation of the daughter nuclei, the chromosomes gradually surround the 'polar masses' (figures 106 to 108), and a ring consisting of chromatic material is formed round each 'polar mass' (figures 109, 110). Later on the chromatic material fragments to give rise to granules as seen in a resting nucleus; stages which are very clear in Feulgen preparations. In iron-alum haematoxylin preparations chromatic material surrounding the 'polar masses' could not be seen (figures 126 to 129). The division of the amoeba into two is shown in figures 128 to 131.

No polar caps or centrioles could be seen during any stages of nuclear division.

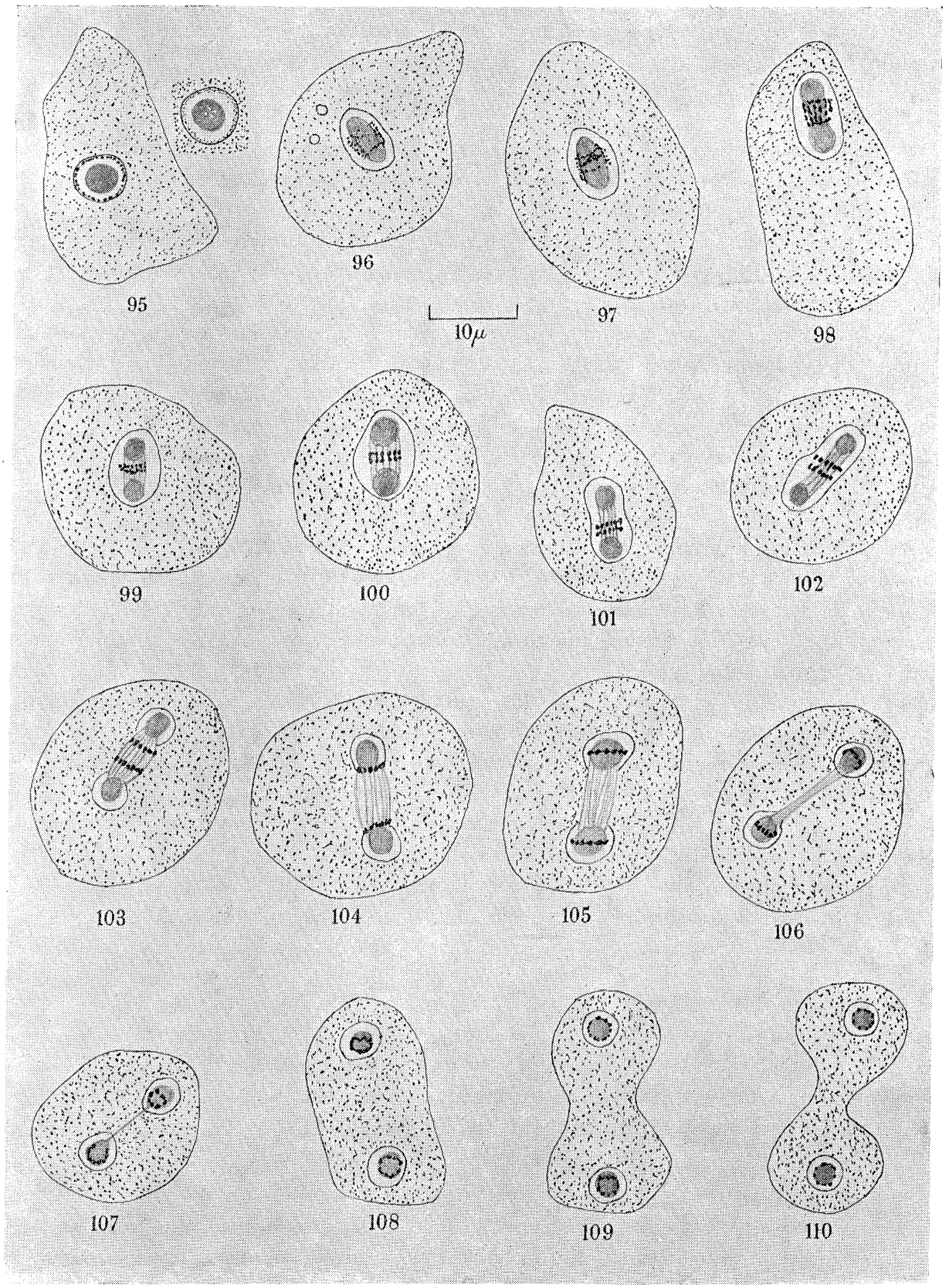
Remarks

Schizopyrenus erythaenusa differs from *S. russelli* in the following main features: (1) Under controlled cultural conditions the trophic forms of *S. erythaenusa* are larger than *S. russelli*. (2) The cysts of the two species are easily distinguishable. (3) The chromosomes, in Feulgen preparations, at metaphase and anaphase can easily be counted in *S. erythaenusa* while they are not clearly distinguishable in *S. russelli*.

(5) *Schizopyrenus atopus** n.sp.

The amoeba was isolated once from Barnfield (plot 4) complete minerals + sulphate of ammonia-treated soil from a dilution of 1/1600, and on another occasion from Barn-

* This species has been created on the basis of odd behaviour of the amoeba when grown on non-nutrient agar plates supplied with *Aerobacter* sp. as food. (Greek ἄτοπος, 'odd, unusual'.)



FIGURES 95 to 110

field (plot 8.0) untreated soil from a dilution of 1/400. These observations suggest that this species may not be present in large numbers in Rothamsted soils.

Morphology, etc.

The active and trophic form of *Schizopyrenus atopus* are indistinguishable from *S. erythaenusa*. It differs from the latter in the character of the cysts. The amoebae are usually uninucleate, although individuals having two nuclei have been seen on many occasions.

The living cysts are very variable in size and are rounded or spherical. They have a single wall (figure 145*c*, *d*). The outside of the wall consists of a transparent gelatinous layer as described in the case of *Didascalus thorntoni*. The nucleus with its nucleolus is very clearly seen inside a living cyst. No chromatic granules could be distinguished. After being in culture for a few months on *Aerobacter* sp., the amoebae lose the power of forming mature cysts and behave very abnormally as will be described later on. On several occasions it was thought that a culture of a few weeks old had died, but on subculturing active amoebae were obtained. On repeated subculturing after every 2 to 3 days a few cysts which appeared to be mature could be obtained. In this connexion it may be of interest to mention that in *Leptomyxa reticulata* Goodey, the organisms, after having been in association with *Aerobacter* sp. for about 6 months, completely lose the power to produce cysts (Singh 1948*a*). The ability of *Leptomyxa reticulata* to produce cysts was regained by culturing them on a few strains of bacteria, out of over a hundred, that were tried. No correlation between the edibility of bacteria by *L. reticulata* and cyst formation could be observed (Singh 1948*a*). It would be of interest to find out if *Schizopyrenus atopus* could be induced to produce large numbers of mature cysts in cultures when fed with different bacteria.

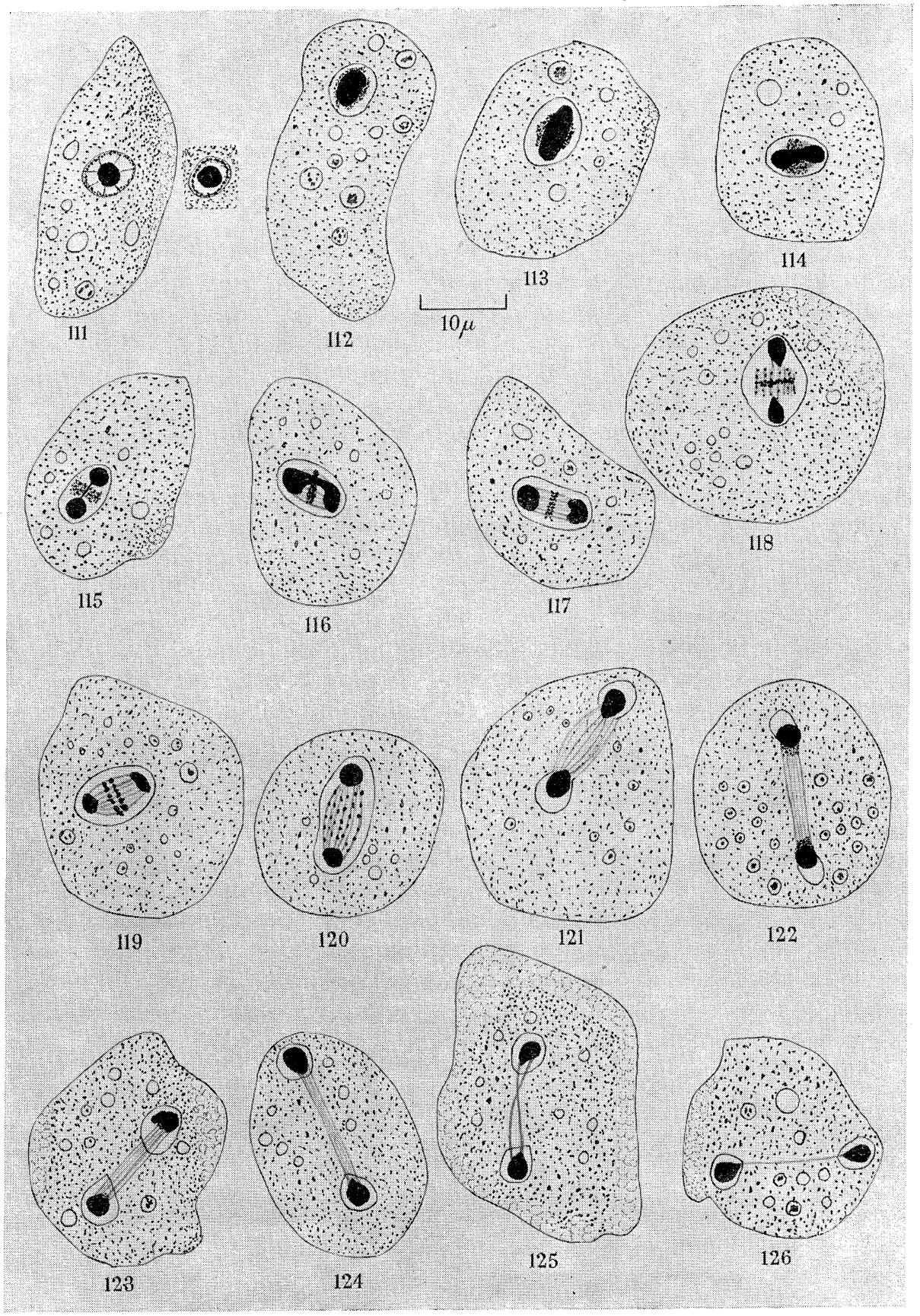
An interesting feature of *S. atopus* is the production of red pigment when grown in association with *Aerobacter* sp. on non-nutrient agar plates. Sometimes the pigment is very faint in colour, and at other times it disappears in culture. On subculturing, the pigment is seen again to be produced by the amoebae. The production of this pigment has been seen over a period of 4 years since the amoeba was first isolated and it was this character that attracted the attention of the writer to isolate the amoeba from a low-dilution culture plate used for the quantitative estimation of soil amoebae (Singh 1949). Although this strain has been isolated several times in 'pure-line' cultures starting from single trophic forms, it has retained the power to produce pigment. One or two other species of amoebae on rare occasions have been found to produce a very faint red pigment but not so pronounced as in *Schizopyrenus atopus* and *S. erythaenusa*.

Efforts to produce a temporary flagellate stage have been completely unsuccessful.

Schizopyrenus erythaenusa. FIGURES 95 to 110. Fixed in Carnoy and stained with Feulgen reaction and light green

FIGURE 95. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 96 to 110. Successive stages in division showing the behaviour of chromatin and the nucleolus.



FIGURES 111 to 126

Structure of the resting nucleus in fresh and in stained preparations

The structure of the resting nucleus (figures 133, 146) is similar to that of *S. erythaenusa*. There is a large Feulgen-negative nucleolus, and Feulgen-positive chromatic granules are present (figure 133).

Mitotic division

The amoebae, after being cultured for several months, behave so abnormally that it becomes exceedingly difficult to get all the stages of normal nuclear division. After careful search and the examination of a very large number of dividing individuals, a more or less complete picture of the normal nuclear division has been presented in figures 147 to 161. This is based on iron-alum haematoxylin preparations. Several stages of the nuclear division from Feulgen preparations are given in figures 134 to 138. As the type of nuclear division is similar to that found in *S. erythaenusa*, it is not intended to repeat these descriptions again. The chromosomes are seen at meta- and anaphases in Feulgen preparations. It is difficult to count their numbers with certainty. The spindle fibres connecting the 'polar masses' are very clearly seen both in Feulgen and iron-alum haematoxylin preparations. The nuclear membrane persists throughout nuclear division and constricts to form two daughter nuclei as in the case of amoebae described earlier.

No 'polar caps' or centrioles could be seen at any stage during nuclear division.

Abnormal stages of nuclear division and the so-called exogenous and endogenous budding

Unless great care is taken to look for the normal stages of nuclear division in *S. atopus*, one may describe new ways in which the nuclei divide both by mitosis and amitosis as has been claimed by Zulueta (1917) in *Wasielewskia gruberi* and Aragão (1909) in *Amoeba diplomitotica* and others. It is not intended to review the literature on the very peculiar ways in which the nuclei in free-living and parasitic amoebae have been claimed to divide by earlier workers, when using iron-alum haematoxylin preparations. It may be pointed out, however, that in *Schizopyrenus atopus*, by using a little imagination, one can describe several ways in which the nucleus of an abnormal amoeba divides. In figure 162 a stage in the abnormal nuclear division is given which could be interpreted as showing that an amoeba divides by amitosis. This preparation was made with iron-alum haematoxylin and does not show any chromatic granules or chromosomes but only the nucleolus (karyosome) dividing in an abnormal way. Stages similar to figure 162 are very common in *S. atopus*.

In abnormal amoebae stages resembling the exogenous budding of an amoeba, as claimed by Wilson (1916) in *Naegleria gruberi*, can very often be seen. Feulgen staining did not show any Feulgen-positive chromatic granules in these buds as claimed by Wilson (1916) by the use of iron-alum haematoxylin. In figures 140 to 144 the different stages in the digestion of a small amoeba by *Schizopyrenus atopus* are shown which resemble the

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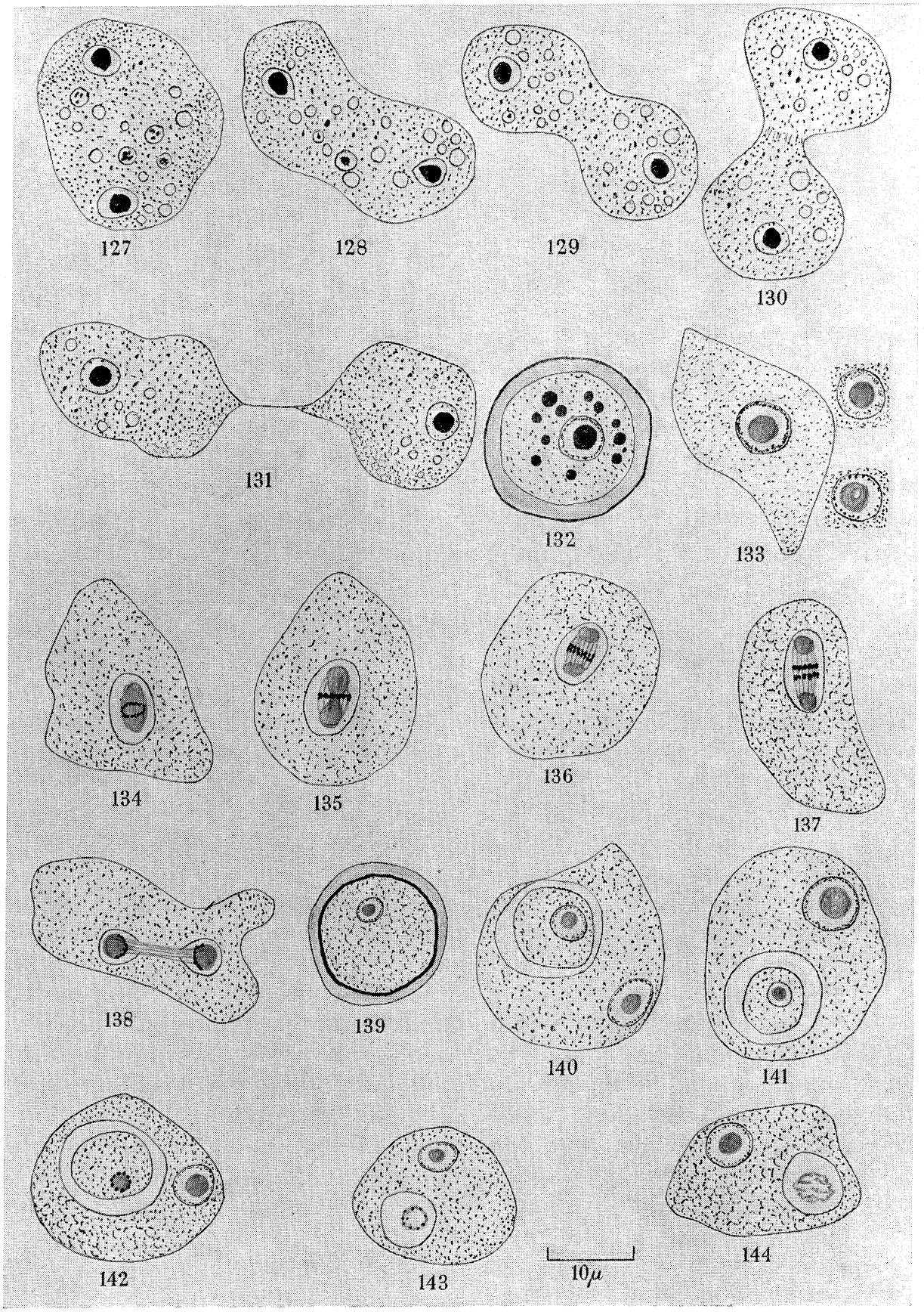
Schizopyrenus erythaenusa. FIGURES 111 to 126. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 111. Ordinary individual and the structure of two resting nuclei.

FIGURES 112 to 126. Successive stages in division.



FIGURES 127 to 144

endogenous budding of Wilson (1916) in *Naegleria gruberi*. As these figures were drawn from Feulgen preparations, there is no sign of the extrusion of chromatic granules from the nucleus, which are supposed to aid in the formation of endogenous buds. The nucleus of the digested amoeba seems to disintegrate, leaving behind certain granular material resembling the formation of endogenous buds.

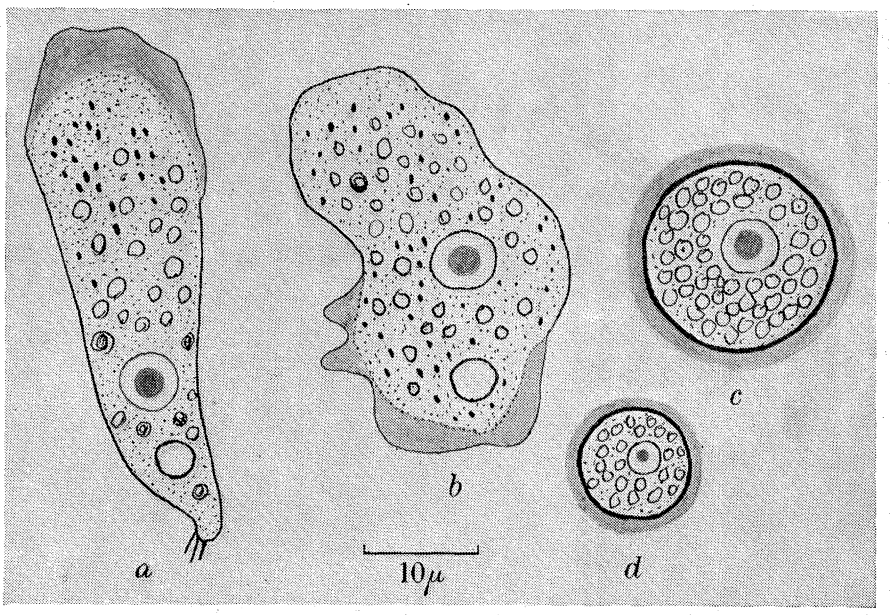


FIGURE 145. *Schizopyrenus atopus*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts.

(6) *Hartmannella glebae* (Dobell)

This amoeba was isolated once from Barnfield (plot 4A) complete minerals+sulphate of ammonia soil from a soil dilution of 1/25600. Although it resembles very closely both *Amoeba lamellipodia* and *A. glebae* described respectively by Gläser (1912*a*) and Dobell (1914), the latter name is used in this work. *A. lamellipodia* is said to possess up to four

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Schizopyrenus erythaenusa

FIGURES 127 to 131. Stages in division. Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURE 132. A cyst of *Schizopyrenus erythaenusa* showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus. Fixed in Carnoy and stained with Feulgen reaction and light green.

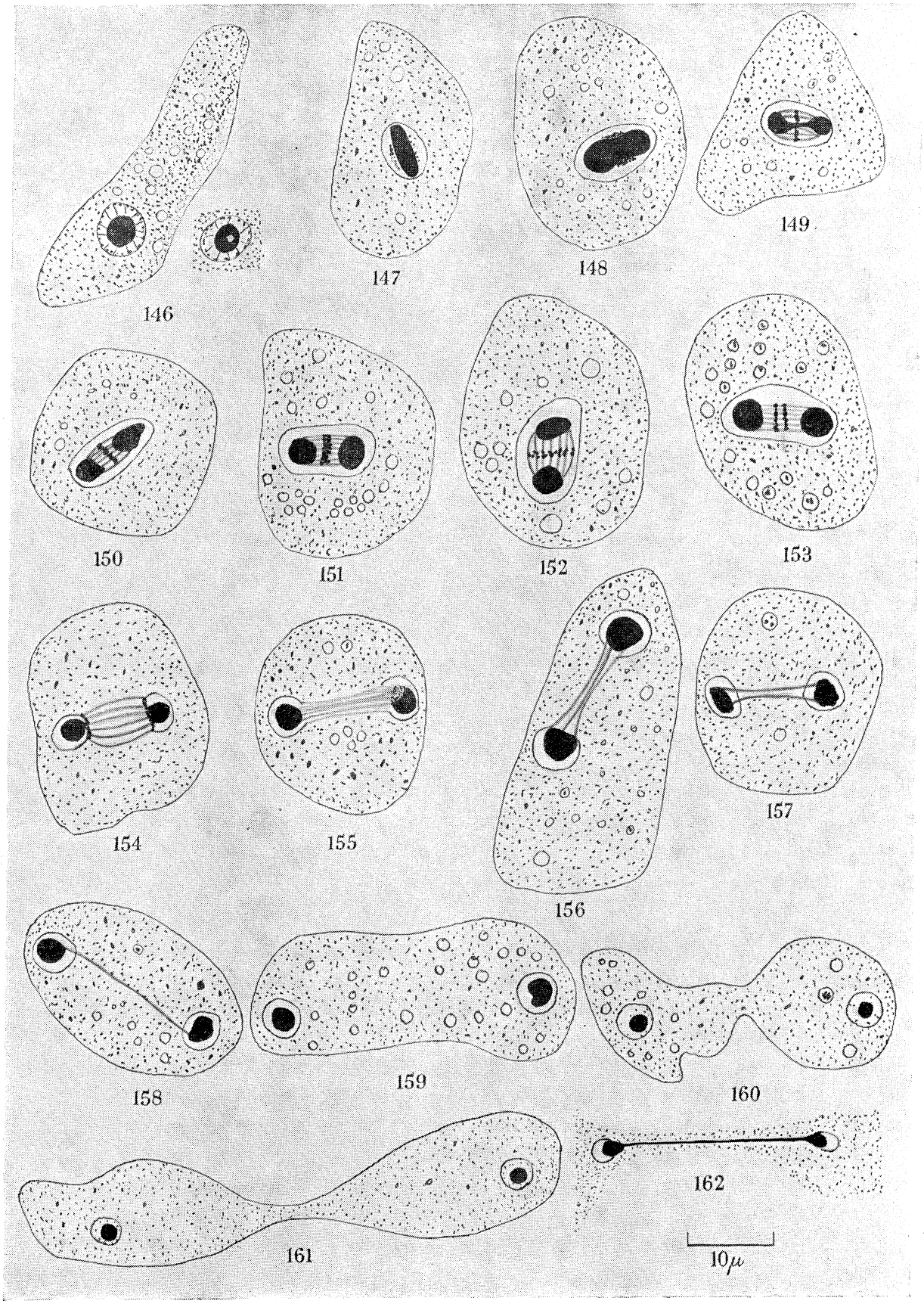
FIGURES 133 to 144. *Schizopyrenus atopus*. Fixed in Carnoy and stained with Feulgen reaction and light green.

FIGURE 133. Ordinary individual and three resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 134 to 138. Stages in division showing the behaviour of chromatin granules and the nucleolus.

FIGURE 139. A cyst showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus.

FIGURES 140 to 144. Stages in the digestion of a small amoeba resembling endogenous bud formation.



FIGURES 146 to 162

contractile vacuoles. The organism studied by the writer has only one such structure as noted by Dobell (1914). This is one of the species of amoebae belonging to the new family Hartmannellidae. It has been put in the genus *Hartmannella* Alexeieff em. Aut.

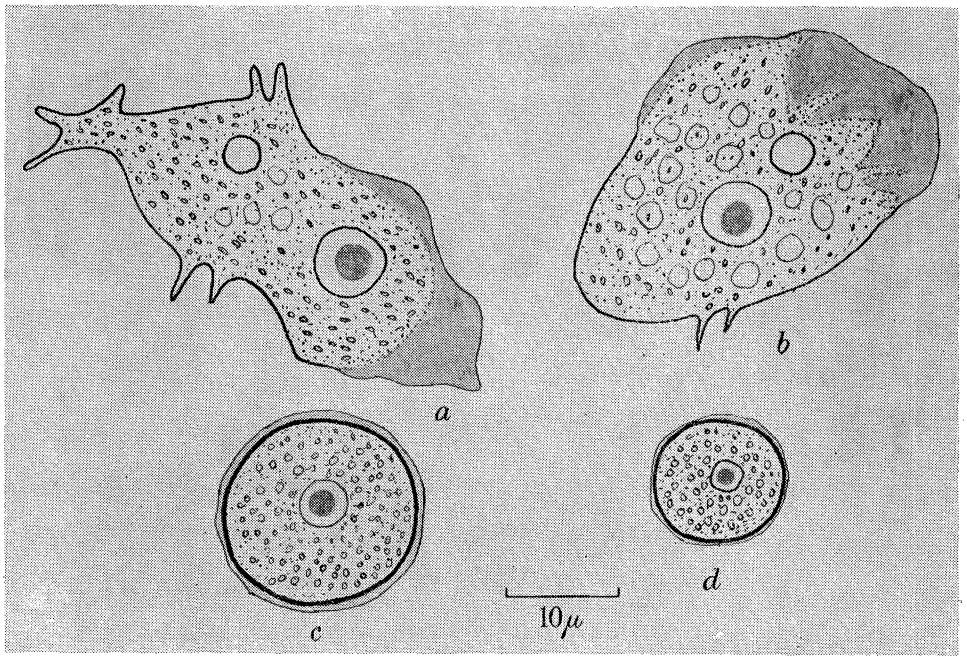


FIGURE 163. *Hartmannella glebae*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts.

Morphology, etc.

The amoebae are very variable in size and in shape. In a rounded condition they are approximately 15 to 30μ across. *Amoeba glebae*, according to Dobell (1914), has a size of 12 to 20μ. The small size of Dobell's organism is probably due to the fact that he used rich nutrient liquid media and uncontrolled bacterial food supply. The species of amoebae described in the family Schizopyrenidae have been found to be very active in young culture and are readily able to produce the *limax* shape. *Hartmannella glebae* and other species described in this genus are not very active and rarely produce the *limax* shape. The movement of *Amoeba lamellipoda*, as described by Gläser (1912*a*) applies to *Hartmannella glebae*. The ectoplasm and endoplasm are very clear (figure 163*a*,*b*). The endoplasm is usually full of food vacuoles containing bacteria. The amoebae are uninucleate, although an individual with two nuclei has been seen on several occasions. There is a single contractile vacuole.

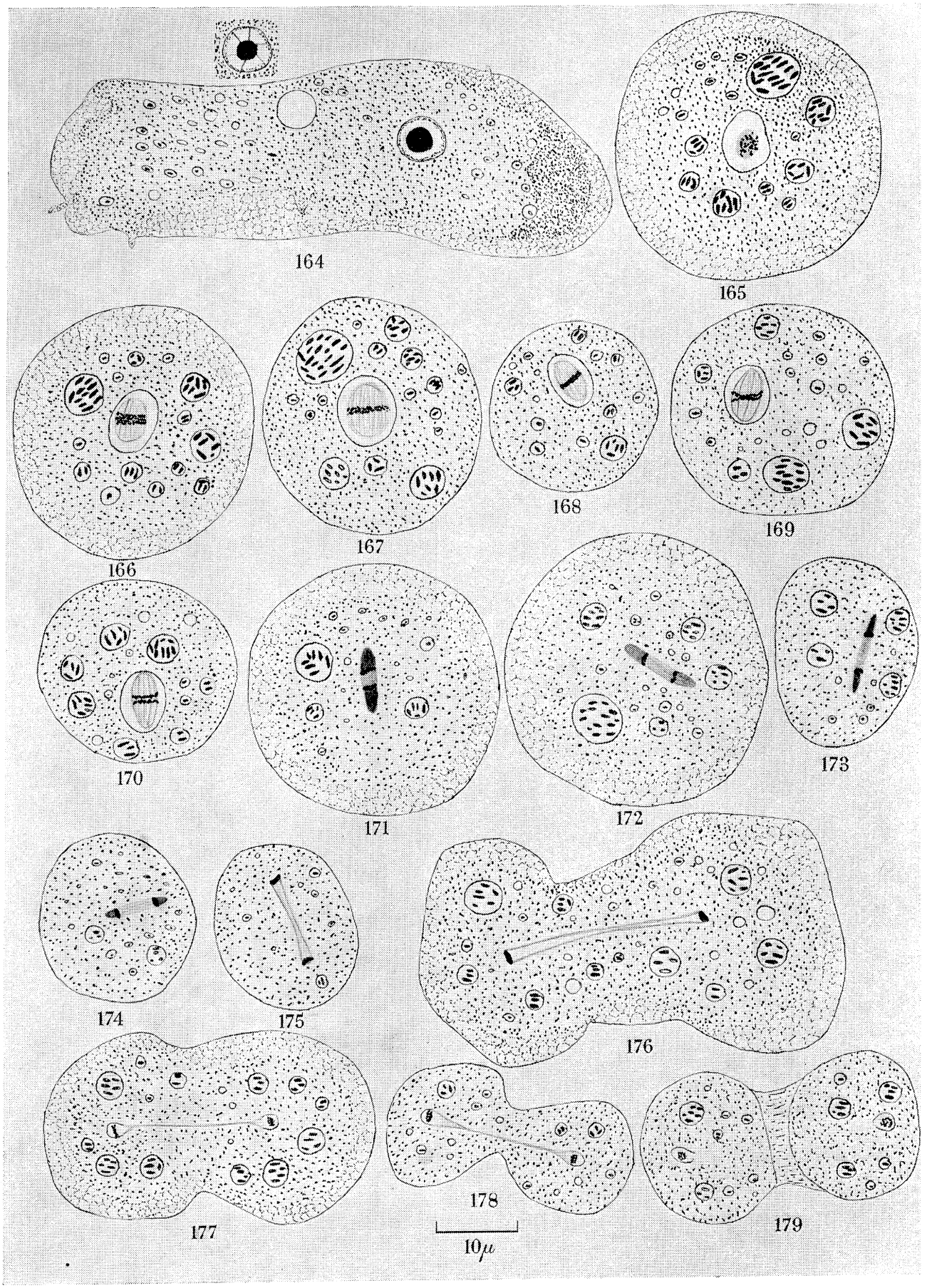
The living cysts are rounded or spherical with a single wall (figure 163*c*,*d*). The wall seems to consist of two layers in which no pores could be seen. The nucleolus is clearly seen in the nucleus of a living cyst, but no chromatin granules could be distinguished.

Schizopyrenus atopus. FIGURES 146 to 162. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 146. Ordinary individual and the structure of two resting nuclei.

FIGURES 147 to 161. Successive stages in division.

FIGURE 162. An abnormal stage in division.



FIGURES 164 to 179

No flagellate stage could be produced by the methods described in connexion with amoebae having a temporary flagellate stage.

Structure of the resting nucleus in fresh and in stained preparations

The structure of the resting nucleus (figure 163*a*, *b*, figures 164, 183) is similar to that described in the species belonging to the genus *Schizopyrenus*. In Feulgen preparations the resting nucleus consists of a Feulgen-negative nucleolus (karyosome) and Feulgen-positive red chromatin granules situated usually near the nuclear membrane (figure 183).

Mitosis

The behaviour of the Feulgen-positive chromatin and Feulgen-negative nucleolus is shown in figures 183 to 200. The amoebae become rounded and motionless at the beginning of the nuclear division. In this respect *Hartmannella glebae* and two other species of this genus differ from all the amoebae described, belonging to the family Schizopyrenidae. The ectoplasm and endoplasm can be clearly seen in some of the rounded individuals (figures 165, 166, 171 and others).

Prophase. In the early stages in the process of nuclear division the Feulgen-positive chromatic granules move to the centre (figure 184). This gives the appearance of the fragmentation of the nucleolus giving rise to chromatic granules, as claimed by Dobell (1914) and Gläser (1912*a*), when using iron-alum haematoxylin preparations as the main criterion to distinguish between chromatic and non-chromatic substances. Both the red-coloured chromatic granules and the green-coloured nucleolus can be easily distinguished without any confusion by Feulgen reaction counterstained with light green (figures 184, 185). The small chromatic granules fuse together to give rise to larger granules. A coiled thread with deeply staining red granules like beads can be clearly seen (figures 185, 186). This resembles the spireme stage. It is not possible to count the number of beads with certainty. On some occasions the thread appears to have free ends (figure 186), and on others it is a closed loop (figures 187, 188). The nucleolus gradually disappears, probably giving rise to the spindle. The spindle is somewhat globular in the early stages with a loop of Feulgen-positive material at its centre (figures 187, 188).

Metaphase. The loop of chromatic material assumes the shape of a solid band at the equatorial plate stage (figure 189). No chromosomes could be distinguished. The band divides into two, and each part appears granular in structure (figure 190). The division of a chromosome into halves could not be seen.

Anaphase. The globular spindle gradually becomes very elongated and thinner (figures 192 to 194, 171 to 174). The fibres in the spindle are not very distinct and fuse when the spindle becomes elongated. At anaphase the nuclear membrane disappears, although in some dividing amoebae it seems to disappear earlier. The parts of the spindle which lie between the chromosomes and the poles stain more deeply and appear like caps. They become smaller and smaller as the chromosomes move to the two poles

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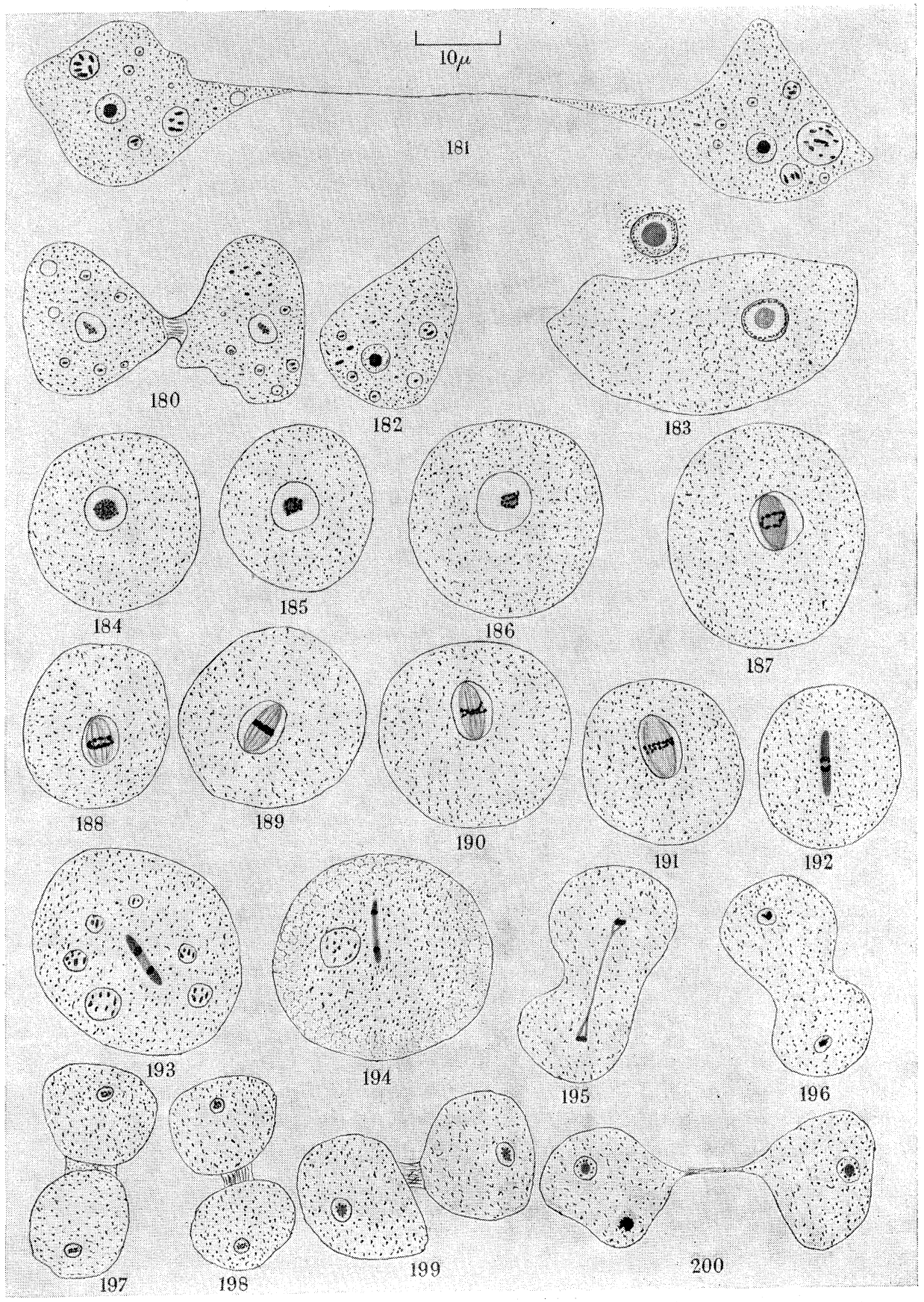
Hartmannella glebae. FIGURES 164 to 179. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 164. Ordinary individual and the structure of two resting nuclei.

FIGURES 165 to 179. Stages in the division.



FIGURES 180 to 200

(figures 193, 194, 171 to 174) and finally disappear. When the chromosomes reach the two ends of the poles, they seem to be connected by a thread-like structure very narrow in the middle (figures 195, 176). The shape of the amoeba becomes elliptical (figures 195, 176).

Telophase. A constriction appears in the middle of the elongated amoeba and two daughter individuals are produced (figures 195 to 200, 176 to 181). A strand of protoplasm connects the two daughter amoebae before they separate (figures 180, 181, 199, 200). Pseudopodia are usually seen to be formed in the later stages of the separation of the two daughter amoebae.

At the stages shown in figures 177 to 179, 196, nuclear membranes appear surrounding each lump of chromatic material. This material fragments into granules (figures 197 to 200), and a pale-staining Feulgen-negative nucleolus is gradually formed (figures 199, 200). The writer is unable to throw any light on the origin of daughter nucleoli. The granular chromatic material later on occupies the position as seen in a resting nucleus (figures 200, 181).

It may be emphasized that there is no aster, centrosome—intra-nuclear or extra-nuclear—at any stage in the mitosis of *H. glebae*, as pointed out by Dobell (1914).

Critical remarks

Gläser (1912*a*) in *Amoeba lamellipodia* and Dobell (1914) in *A. glebae*, by the use of iron-alum haematoxylin staining, claimed that the chromatic material arises from the fragmentation of nucleolus (karyosome). Dobell (1914) says (p. 161): 'The form of the resting nucleus is very characteristic. It contains a large central karyosome, consisting apparently of chromatic granules imbedded in a plastin matrix, and surrounded by a clear zone which separates it from the very delicate nuclear membrane—the latter having a slightly beaded appearance. Fine radiating strands of linin pass from the karyosome to the membrane; and upon these—in the clear zone—there is a single layer or zone of palely staining granules, which appear in optical section as a ring surrounding the karyosome. The granules themselves would probably be described as "peripheral chromatin" by many writers: but they are composed of a substance different from the chromatin in the karyosome, and—as I shall show—they play no part in nuclear division.'

In the opinion of the writer *A. lamellipodia* and *A. glebae* are forms very closely related. The size differences in these amoebae, as noted by Dobell (1914) and Gläser (1912*a*), are probably due to the cultural conditions and food supply. Without going into the details of the behaviour of chromatic and non-chromatic materials as depicted by Dobell (1914) during the various phases of nuclear division and in the formation of the daughter nucleoli,

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Hartmannella glebae

FIGURES 180 and 181. Stages in division.
FIGURE 182. An amoeba which has just divided.
} Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURES 183 to 200. Fixed in Carnoy and stained with Feulgen reaction and light green.

FIGURE 183. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 184 to 200. Successive stages in division. Feulgen-positive chromatic granules give rise to chromosomes and the nucleolus disappears.

it is sufficient to point out that the writer is not in agreement with his findings. The exclusive use of iron-alum haematoxylin staining gives such a misleading picture of the chromatic and non-chromatic materials in prophase and telophase stages of nuclear division that while using this method and before Feulgen reaction and the light green counterstain were tried, the writer fully agreed with the views of Dobell (1914). The views of Gläser (1912*a*) and Dobell (1914), put forward with great emphasis, have had a considerable effect on many protozoologists studying the same type of nuclear division. Martin & Lewin (1914) in *A. cucumis* and *A. globanniensis*, Goodey (1916) in *A. lawesiana* and others have without hesitation accepted the theory of the fragmentation of nucleolus (karyosome) to give rise to chromosomal chromatin.

By the use of Feulgen reaction Zuelzer (1927) in *Hartmannella biddulphiae* n.sp., Volkonsky (1931) in *H. castellanii* n.sp. have shown that Feulgen-positive chromatic granules are present in the resting nuclei of these amoebae. During mitosis these granules give rise to chromosomes. The nucleolus is Feulgen-negative. In *H. glebae* and three other species belonging to the same genus, it has been shown that there are pre-existing Feulgen-positive chromatic granules in the resting nuclei. During mitosis the chromatic granules move to the centre, the region occupied by the nucleolus, and give rise to chromosomes. The Feulgen-negative nucleolus disappears and probably gives rise to the spindle. The question of the formation of the spindle from the nucleolus will be discussed in connexion with the work on *H. agricola* described later on.

The amoebae belonging to *Hartmannella* have been subdivided by Arndt (1921), Nöller (1922), Wenyon (1926) and Volkonsky (1931), but in the present state of our knowledge of the amoebae belonging to this genus, it is not possible to divide it further. Wenyon (1926) distinguished two types: the one characterized by a rounded spindle during nuclear division and cysts possessing a smooth outer surface, and the other by a pointed spindle and a wrinkled outer layer of the cyst. These characters are not sufficiently weighty to justify dividing the genera into groups or subgenera. The four species of this genus described in this work have been found to possess a rounded spindle during early stages of nuclear division, although the cysts of some of them are smooth and of others wrinkled. Moreover, the rounded and pointed spindles are not very well defined in some species. The rounded, cylindrical and pointed spindles in conjunction with morphological and other characters may be a useful means of recognizing species. Further careful work, under controlled and reproducible cultural conditions, is urgently needed before valid species of the genus *Hartmannella* can be recognized.

As suggested by Volkonsky (1931) the amoebae, like *Amoeba mira* (Gläser 1912*b*) and *Hartmannella testudinis* (Ivanić 1926), where the nuclear division takes place in the cyst, should be taken out of the genus *Hartmannella* and placed in a separate genus (*Gläseria*). The mode of nuclear division places the genus *Gläseria* in the family Hartmannellidae.

(7) *Hartmannella rhysodes** n.sp.

This species has been found in soils receiving farmyard manure, complete minerals + sulphate of ammonia and no manure in Barnfield (plots 1.0, 4A and 8.0) and Broadbalk

* This species has been named from the wrinkled appearance of the majority of the cysts (figure 201). (Greek ῥυσώδης, 'wrinkled-looking'.)

field (plots 2, 7 and 3) at Rothamsted. During the quantitative studies of the amoebic population (Singh 1949) from 1945 to 1948, it was found to be present in large numbers in all the soils. In farmyard manure and complete minerals + sulphate of ammonia soils, *H. rhysodes* has been isolated from the highest soil dilution used, i.e. at 1/1638400, on several occasions. It seems to be one of the most common soil forms at Rothamsted.

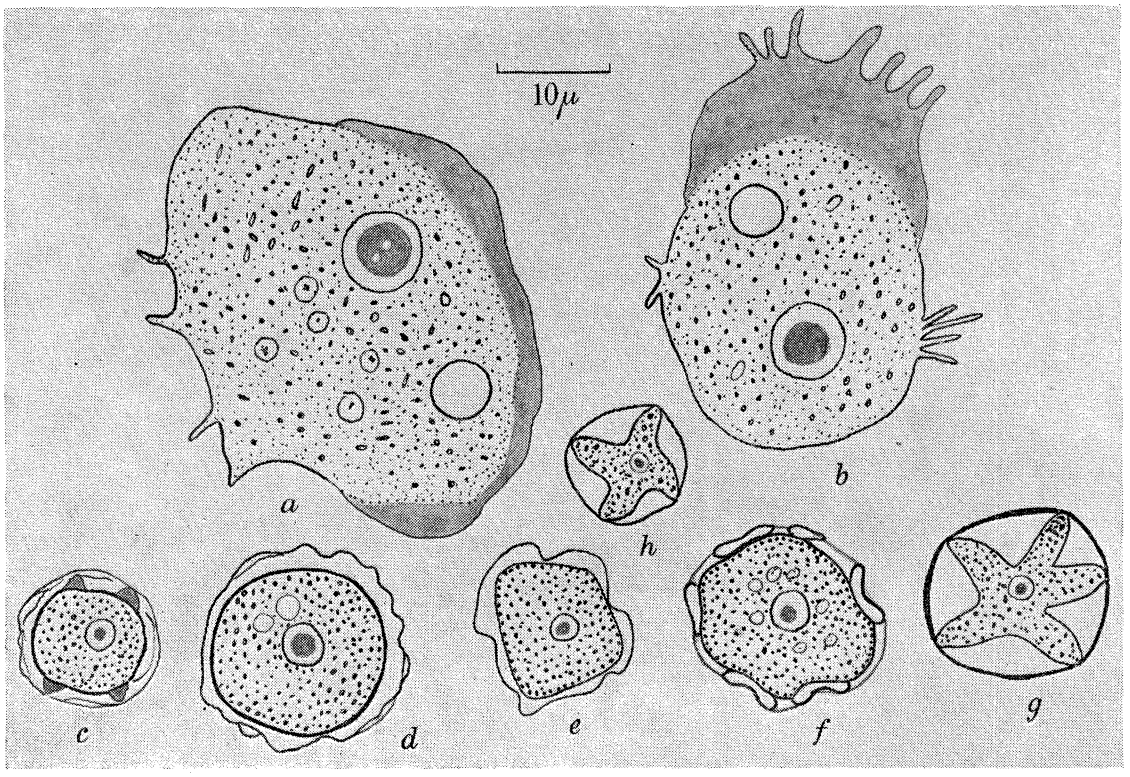
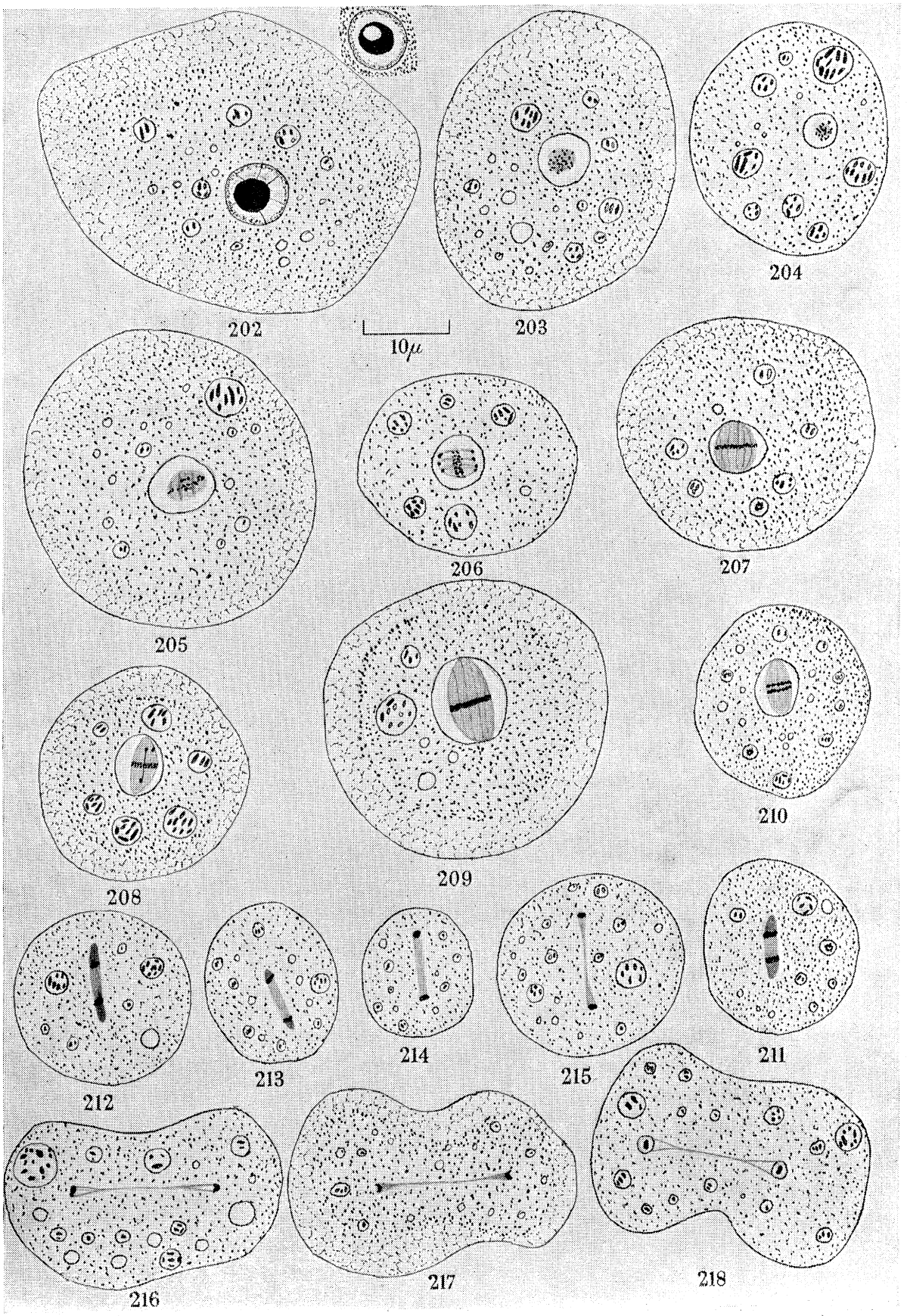


FIGURE 201. *Hartmannella rhysodes*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, *e*, *f*, *g*, *h*, cysts.

Cutler, Crump & Sandon (1922), in the daily count of the bacterial and protozoan population of Barnfield farmyard manured soil, extending over a period of one year, found *Naegleria gruberi* to be a dominant species of amoeba. It is interesting to note that *N. gruberi*, as a dominant soil form, has now been completely replaced by other species. The writer has not been able to isolate *N. gruberi* from Rothamsted soils during a period of over 10 years.

As *Hartmannella rhysodes* produced wrinkled cysts with pores similar to *Naegleria gruberi*, it was thought to be the latter form several years ago. The amoeba used in edibility tests, recovery of counted suspensions, etc., in connexion with the work on a method of estimating the numbers of soil Protozoa (Singh 1946), and called *N. gruberi*, was, in fact, *Hartmannella rhysodes*.

Four strains of *H. rhysodes* (strains 1, 9, 14 and 15) which from the character of their cysts appeared different at first sight, were isolated in 'pure-line' cultures, starting from single cysts. The cysts, however, are so very variable within each strain that their appearance cannot safely be used as a diagnostic character. In figure 201 are given several cysts which appear different. As no differences in the nuclear division of these four strains have



FIGURES 202 to 218

been found, it is proposed at present to consider them as a single species. Some minor differences in the morphology and other characters of two or three strains have been found, but they do not seem to justify the creation of species. Further studies of these strains under standardized cultural conditions and serological tests may be interesting in determining whether they are different biological races or even two or three distinct species.

Morphology, etc.

The amoebae have very variable size and shape. In a rounded condition they are approximately 15 to 35μ. In general appearance the active and trophic amoebae appear similar to *H. glebae*. They move very slowly and rarely produce the *limax*-like form (figure 201*a*,*b*). The movement is similar to *H. lamellipodia* described by Gläser (1912*a*). The ectoplasm and endoplasm are very distinct. Thin protoplasmic prolongations are very often seen (figure 201*b*). The amoebae are uninucleate, although two or three nuclei have been found in an individual. There is a single contractile vacuole.

The living cysts are very variable in size and shape (figure 201*c*,*d*,*e*,*f*,*g*,*h*). Each cyst consists of two walls which are generally irregular in outline and give a wrinkled appearance. On careful examination some of the cysts appear to be pierced by four or more pores. These pores seem to be plugged with some kind of structureless substance as is the case in the cysts of *Naegleria gruberi*. The amoeba comes out of one of these pores during the process of excystment. Ostioles as described by Volkonsky (1931) in *Hartmannella castellanii* could be found in some cysts. Some cysts appear rounded with the contents of the inner cyst wall shrunken, presenting a polyhedral appearance (figure 201*g*,*h*). They resemble to some extent the cysts of *Amoeba albida* described by Nägler (1909), but the latter, according to Nägler (1909), has 'polar masses' during nuclear division. In a living cyst the nucleolus in the nucleus is very distinct, but no chromatin granules could be made out.

Efforts to produce a temporary flagellate stage have completely failed.

Structure of the resting nucleus in fresh and in stained preparations

The structure of the resting nucleus (figures 201*a*,*b*, 202, 223) is similar to that of *Hartmannella glebae*. Feulgen-negative nucleolus and Feulgen-positive chromatin granules are clearly seen in Feulgen preparations counter-stained with light green. One or more non-stainable patches can be seen in some nucleoli (figures 202, 223).

Mitosis

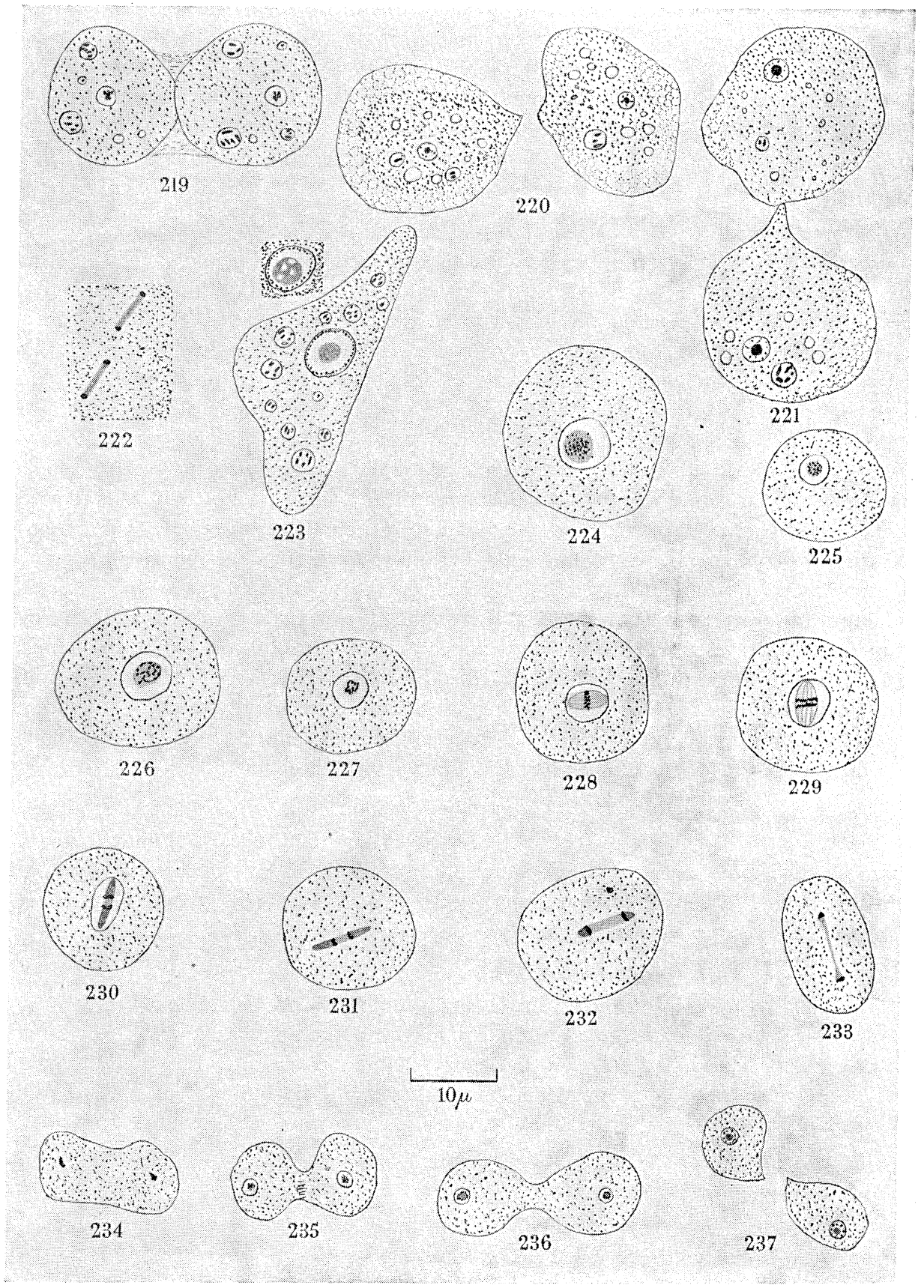
The nuclear division in four strains (1, 9, 14 and 15) of *H. rhysodes* was studied using both Feulgen reaction and iron-alum haematoxylin preparations. No differences in any of these strains could be found, and the figures 202 to 237 are drawn from strain 9.

The amoebae become rounded and motionless at the beginning of nuclear division. The ectoplasm and endoplasm can be clearly seen in some rounded individuals (figures 203, 205, 207, 209).

Hartmannella rhysodes. FIGURES 202 to 218. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 202. Ordinary individual and the structure of two resting nuclei.

FIGURES 203 to 218. Successive stages in division.



FIGURES 219 to 237

As the nuclear division resembles *H. glebae* in many respects, only a brief description of the stages of mitosis is given below.

Prophase. The Feulgen-positive chromatin granules move to the centre (figures 224, 225). This gives the appearance of the fragmentation of the nucleolus in iron-alum haematoxylin staining (figures 203, 204). The small chromatic granules fuse to give rise to a coiled thread with deeply red-staining Feulgen-positive granules like beads (figures 226, 227). The nucleolus gradually disappears and probably gives rise to the spindle.

Metaphase. The chromosomes at the equatorial plate, in Feulgen preparations, could not be counted nor could the division of each chromosome into two be made out (figures 228, 229).

Anaphase. The globular spindle gradually becomes elongated and thinner (figures 230 to 232, 211 to 213). The elongated spindle in this amoeba is slightly pointed compared with that in *H. glebae.* The nuclear membrane disappears either at anaphase or in some cases earlier. The parts of the spindle which lie between the chromosomes and the poles stain more deeply. They become smaller and smaller as the chromosomes move to the poles (figures 230 to 232 and others). The spindle connecting the solid lump of chromosomes at the two poles is very narrow in the middle (figures 215 to 218, 233).

Telophase. A constriction appears in the middle of the elongated amoeba, and the two daughter individuals are produced (figures 216 to 218, 234 to 237). A strand of protoplasm connecting the two amoebae, as seen in *H. glebae*, could not be seen. Probably the amoebae separate very quickly. A nuclear membrane appears surrounding each lump of chromatic material at the two poles (figures 218, 219, 235). Later on the lump fragments to give rise to chromatic granules. These granules gradually move near the nuclear membrane as seen in resting nuclei (figures 235 to 237). The way the daughter nucleoli are formed could not be ascertained.

The nuclear division in amoebae having two nuclei takes place quite normally. A stage in the nuclear division of such an amoeba is shown in figure 222 possessing two nuclei.

Centrosome and aster, etc. An aster and centrosome could not be found at any stage in the mitosis of *H. rhysodes.* In figure 208 a stage is given in which, according to some workers, two centrosomes connected by a thread would seem to be present. In this figure two granules at one end are shown, although only one is connected by a thread-like structure. In another amoeba (figure 206) three to four granules are shown at each end of the spindle in an iron-alum haematoxylin preparation. Stages like this, where more than one granule is present at each pole of the spindle, have been seen in a number of amoebae. This makes

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Hartmannella rhysodes

FIGURES 219 to 221. Stages in division.
FIGURE 222. A stage in the nuclear division of an amoeba having two nuclei.
} Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURES 223–237. Fixed in Carnoy and stained with Feulgen reaction and light green.

FIGURE 223. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 224 to 237. Successive stages in division. Feulgen-positive chromatic granules give rise to chromosomes and the nucleolus disappears.

the writer very doubtful of the presence of centrioles in the nuclear division of amoebae as stressed by Dobell (1914) and others. These granules are probably some kind of non-chromatic material arising from the nucleolus during the process of its disappearance.

(8) *Hartmannella leptocnemus** n.sp.

This amoeba was isolated once from (plot 3) untreated soil of Broadbalk at a dilution of 1/25600 and on another occasion from Barnfield (plot 4A) complete minerals + sulphate of ammonia soil at a dilution of 1/1638400. It seems to be a common soil form at Rothamsted.

Morphology, etc.

The amoebae in a rounded condition are 10 to 20μ across. They do not usually produce the *limax* shape and move very slowly. The body presents a great variety of sizes and shapes and the pseudopodia are irregular and lobose. The individuals often become elongated and have a characteristic shape (figure 238*b,c*). There is no clearly defined ectoplasm and endoplasm even when the amoebae move. Several vacuoles resembling contractile vacuoles are present in each amoeba (figure 238*a,b,c,d*). These vacuoles have not been seen to contract. Amoebae are uninucleate, although an amoeba with two nuclei could be seen occasionally.

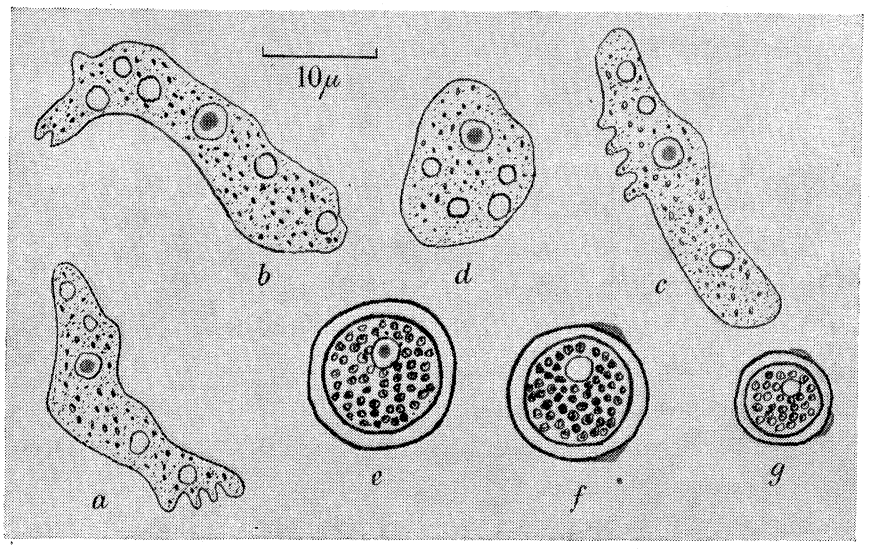


FIGURE 238. *Hartmannella leptocnemus*, drawn in the living condition.
a, b, c, d, trophic forms; *e, f, g*, cysts.

The living cysts are rounded or spherical with a double wall (figure 238*e,f,g*). They are variable in size and resemble the double-walled cysts of *Schizopyrenus russelli*. The nucleolus in the nucleus could be seen clearly only in some of the living cysts, but no chromatic granules could be made out as seen in Feulgen preparations (figure 261).

No temporary flagellate stage could be produced in *H. leptocnemus*.

* This species has been named from the characteristic elongated appearance of amoebae when grown on non-nutrient agar plates supplied with *Aerobacter* sp. as food (figure 238). (Greek λεπτόκνημος, 'spindle-shanked'.)

Structure of the resting nucleus in fresh and in stained preparations

The structure of the resting nucleus (figures 238*a*, *b*, *c*, *d*, 239, 248) is similar to that in *Hartmannella glebae*. The Feulgen-negative nucleolus and Feulgen-positive chromatin granules are clearly shown in figure 248.

Mitosis

The amoebae become rounded at the beginning of the nuclear division. No differentiation between ectoplasm and endoplasm could be seen.

The behaviour of chromatic granules and the nucleolus are clearly seen in Feulgen preparations (figures 248 to 260). The type of nuclear division is similar to that found in *H. glebae* and *H. rhysodes*.

Prophase. The Feulgen-positive chromatic granules move to the centre. In figure 249 is shown a stage where the granules are situated round the nucleolus. It seems in this amoeba that the nucleolus becomes elongated and gives rise to the spindle (figures 241, 242, 250, 251). In iron-alum haematoxylin preparations the nucleolus seems to lose its staining properties during its transformation into the spindle (figures 241, 242). The spindle fibres are not clearly seen. The spindle is globular in the early stages of its formation and then becomes somewhat pointed (figures 250 to 252). The chromatic granules fuse to give rise to a coiled structure lying in the middle of the nucleolus (figure 250).

Metaphase. A band of granular chromatic material is seen lying at the equatorial plate stage (figure 251). The number of chromosomes could not be counted. The band divides into two (figure 252).

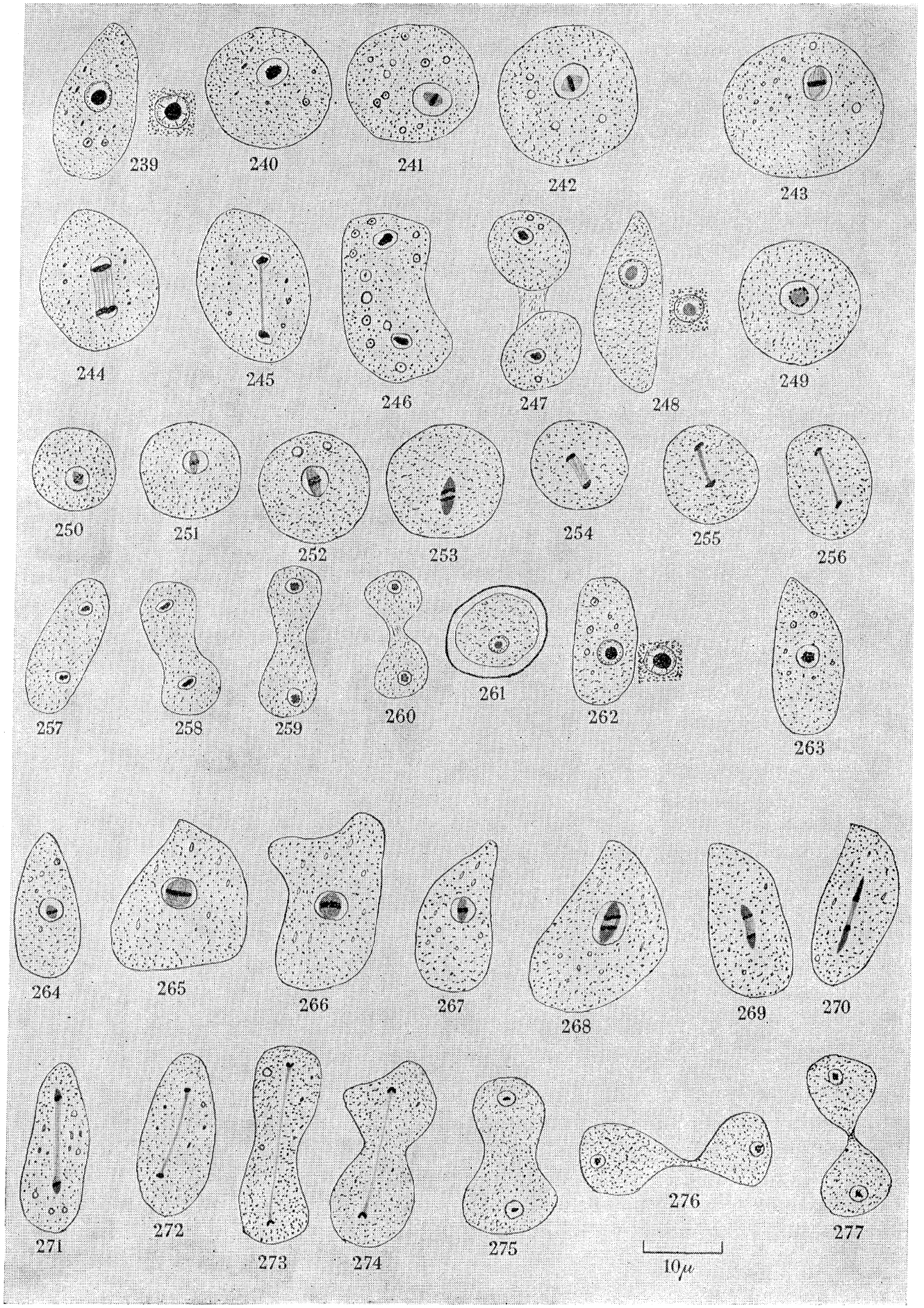
Anaphase. The nuclear membrane disappears at anaphase (figure 253). The two bands of chromosomes move to the two poles. The parts of the spindle which lie between the chromosomes and the poles stain more deeply and become smaller and smaller as the chromosomes approach the poles. The chromosomes at the two poles remain connected by narrow spindle fibres which are not distinct (figures 254 to 256). Finally the spindle disappears.

Telophase. The amoeba becomes elongated and a constriction appears in the middle producing two daughter individuals (figures 246, 257 to 260). At the stages shown in figures 244 to 246, 257 to 260 a nuclear membrane is formed surrounding each lump of chromatic material. The membrane is slightly elongated at first and later on it assumes a rounded outline. The Feulgen-positive chromatic material fragments and a new nucleolus is produced in each daughter nucleus. The chromatic granules occupy the position seen in the resting nuclei. The manner in which the new nucleoli are produced could not be ascertained.

No intra-nuclear or extra-nuclear aster and centrosome could be seen at any stage in the mitosis of *H. leptocnemus*.

Abnormal division stages

The amoebae after living in culture for several months behave abnormally to some extent, as was found in *Schizopyrenus atopus*. The nucleolus often divides so abnormally that great care is needed to find the normal division stages. If there is insufficient material it is easy to mistake the granulation of the nucleolus and various other abnormal stages for a new type of nuclear division as was suggested by Goodey (1916) in *Hartmannella agricola*.



FIGURES 239 to 277

(9) *Hartmannella agricola* (Goodey 1916) n.comb.

Syn. *Amoeba agricola* Goodey 1916.

This form has been obtained in 'pure-line' cultures on three occasions. It was isolated from Broadbalk (plot 2) farmyard manured soil at a dilution of 1/409600, from complete minerals + sulphate of ammonia soil (plot 7) at a dilution of 1/1600 and also from Barnfield untreated soil (plot 8.0) at a dilution of 1/51200. It seems to be a common soil form at Rothamsted. As this amoeba resembles *Amoeba agricola* described by Goodey (1916), it has been called *Hartmannella agricola*.

Morphology, etc.

The amoebae do not usually produce the *limax* form and move very slowly. The elongated active forms (figure 278*a*, *b*, *c*, *d*) resemble *H. leptocnemus*, although they do not assume any very characteristic shape. It is difficult to measure the amoebae as they do not assume a rounded shape. They appear slightly smaller than *H. leptocnemus*. The distinction between ectoplasm and endoplasm is not at all clear either in the living or in the stained amoebae. No contractile vacuole has been seen. The amoebae are uninucleate, but occasionally an amoeba with two or three nuclei has been found.

The living cysts are spherical and very variable in size, having a single-layered wall (figure 278*e*, *f*, *g*). No pores in the cyst wall could be seen. The nucleolus in the nucleus of a living cyst could not be clearly seen.

No temporary flagellate stage has been found in *H. agricola*.

The structure of the resting nucleus in fresh and in stained preparations

The structure of the nucleus (figure 262) is similar to that of *H. leptocnemus*.

Mitosis

The amoebae retain their irregular outline during mitosis. In this respect *H. agricola* differs from the three species belonging to the same genus already described.

Hartmannella leptocnemus

FIGURE 239. Ordinary individual and the structure of two resting nuclei.

FIGURES 240 to 247. Stages in division.

Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURES 248 to 261. Fixed in Carnoy and stained with Feulgen reaction and light green.

FIGURE 248. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 249 to 260. Successive stages in division. Feulgen-positive chromatic granules give rise to chromosomes and the nucleolus disappears.

FIGURE 261. A cyst showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus.

Hartmannella agricola. FIGURES 262 to 277. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 262. Ordinary individual and the structure of two resting nuclei.

FIGURES 263 to 277. Successive stages in division.

Unfortunately, the culture of *H. agricola* was lost and the stages of nuclear division in Feulgen preparation could not be studied. The figures 262 to 277 are drawn from iron-alum haematoxylin stained specimens.

The behaviour of the chromatic granules and the nucleolus (figures 262 to 277) is very similar to that found in *H. leptocnemus*, and it is not intended to repeat the description. The reader can obtain a good idea of the mode of nuclear division by looking at the figures 263 to 277. The nucleolus loses its staining properties very markedly in the process of being converted into the spindle (figure 264) as was found in *H. leptocnemus*. The chromatin granules give rise to the chromosomes and at the end of nuclear division the chromatic mass fragments to give rise to the granules of the resting nucleus. A new nucleolus is formed in nucleus of the daughter individuals.

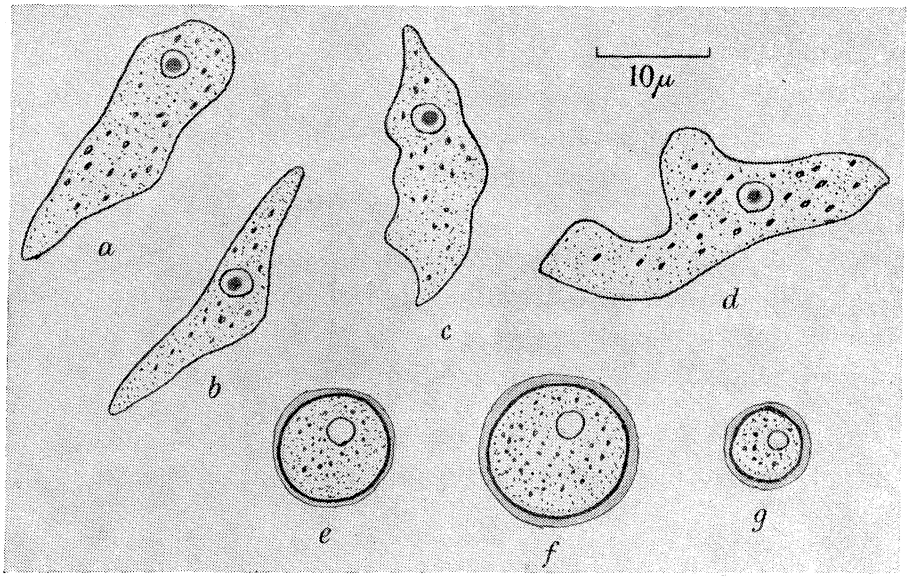


FIGURE 278. *Hartmannella agricola*, drawn in the living condition. *a*, *b*, *c*, *d*, trophic forms; *e*, *f*, *g*, cysts.

The spindle of *H. agricola* differs in some ways from that of *H. leptocnemus*. It seems that in the former the spindle, after being like a globe in the early stages (figures 265, 266), becomes gradually elongated and very pointed (figure 270). The spindle fibres in *H. agricola* are not so distinct as in *H. leptocnemus*.

No intra-nuclear or extra-nuclear aster and centrosome could be found at any stage in the mitosis of *H. agricola*.

Abnormal stages of division

Abnormal stages in nuclear division as shown in figure 162 for *Schizopyrenus atopus* have often been seen in this amoeba. These abnormally dividing amoebae give the appearance of amitosis. It is interesting to note that abnormal behaviour of the nucleolus giving the impression of an amitotic division has been found in amoebae belonging to both the families Schizopyrenidae and Hartmannellidae.

Critical remarks

Goodey (1916) described *Amoeba agricola* n.sp. from one of his cultures made from stored Hoosfield soil at Rothamsted. He says (p. 325): 'It exhibits some rather remarkable

appearances during the division of the nucleus, which seems to differ from any of the already described nuclear divisions in amoebae; and it is on the strength of this fact that I propose the creation of a new species for its reception.'

The amoeba just described resembles Goodey's amoeba in several respects. It has no distinction between ectoplasm and endoplasm, and during nuclear division it retains its irregular appearance as was clearly pointed out by Goodey (1916). Moreover, it tends to divide abnormally, and this tendency would probably have been increased in the cultural conditions used by Goodey, since these were not standardized. I have therefore felt justified in applying the specific name *agricola* to this form. Goodey's organism belongs to the genus *Hartmannella*, because had there been the formation of 'polar masses' during nuclear division he would certainly have noted it.

IV. Systematic position of the amoebae studied

Class Rhizopoda von Siebold
Subclass Amoebaea Bütschli
Order Amoebida Calkins
Family Schizopyrenidae n.fam.
Genus *Naegleria* Alexeieff em. Aut.
Species *Naegleria gruberi* (Schardinger)
Genus *Didascalus* n.g.
Species *Didascalus thorntoni* n.sp.
Genus *Schizopyrenus* n.g.
Species *Schizopyrenus russelli* n.sp.
Species *Schizopyrenus erythaenusa* n.sp.
Species *Schizopyrenus atopus* n.sp.
Family Hartmannellidae n.fam.
Genus *Hartmannella* Alexeieff em. Aut.
Species *Hartmannella glebae* (Dobell)
Species *Hartmannella rhysodes* n.sp.
Species *Hartmannella leptocnemus* n.sp.
Species *Hartmannella agricola* (Goodey) n.comb.

The two new families and the genera are defined in §VI.

V. Discussion and phylogeny

The free-living and parasitic amoebae seem to possess a type of nucleus usually termed 'vesicular' by various protozoologists. Apart from a few species of large free-living amoebae, like *Amoeba proteus*, *Pelomyxa carolinensis* and others, and some parasitic ones like *Endamoeba blattae*, the great majority of amoebae possess a single more or less spherical central nucleolus inside the nucleus. The study of nine species of small free-living amoebae of this type reveals that structurally the resting nuclei are all of the following pattern. There is a Feulgen-negative nucleolus and Feulgen-positive chromatin granules. During mitosis the chromatin granules give rise to chromosomes. The nucleolus either persists throughout division, giving rise to 'polar masses' in forms like *Naegleria gruberi*, *Didascalus*

thorntoni, *Schizopyrenus russelli*, *S. erythaenusa* and *S. atopus*, or it disappears during division as is the case in *Hartmannella glebae*, *H. rhysodes*, *H. leptocnemus* and *H. agricola*. The continuity of the Feulgen-positive chromatin through the various stages of division and its fragmentation at late telophase to give rise to the dispersed chromatin of the resting nuclei has been clearly demonstrated. Similar behaviour of Feulgen-positive chromatin in amoebae possessing a Feulgen-negative nucleolus has been shown by Rafalko (1947) in *Naegleria gruberi*, Zuelzer (1927) in *Hartmannella biddulphiae*, Volkonsky (1931) in *H. castellanii* and others. The work of Kudo (1947), Liesche (1938) and others on *Pelomyxa carolinensis* and *Amoeba proteus* demonstrates that Feulgen-positive chromatin gives rise to chromosomes during division, as has been found in *Hartmanella glebae* and other species belonging to the same genus. Thus it seems that in amoebae that have been carefully investigated by this method the Feulgen-positive chromatin* of the resting nuclei behaves during mitosis in the same way as it does in higher animals and plants.

It seems that one of the chief functions of the nucleolus is the formation of the spindle in all the species of amoebae studied; this is clear in forms like *H. leptocnemus* and *H. agricola*. Structurally the resting nuclei of *Amoeba proteus*, *Pelomyxa carolinensis* and other larger forms seem to be similar, though they contain several nucleoli instead of a single nucleolus. Kudo (1947) has clearly shown that in *P. carolinensis* Wilson the spindle arises from Feulgen-negative nucleoli.

Vahlkampf (1905), Gläser (1912*a*), Dobell (1914) and others in *Amoeba limax*, *A. platypodia*, *A. lacertae* and other forms have claimed that apart from the nucleolus (karyosome) there are no chromatin granules present in the resting nuclei. It is quite possible that the chromatin granules in these forms are in such a finely dispersed state in the resting nuclei that they are easily decolorized in the process of differentiation in iron-alum haematoxylin preparations. Such statements on the absence of chromatin granules in the resting nucleus of an amoeba cannot be accepted unless the Feulgen reaction has been carefully used.

In order to avoid confusion between Feulgen-positive chromatin and other non-chromatic material in a resting nucleus, it is suggested that the term 'peripheral chromatin' should be abolished. This term has been used in the past to include all the granular material in the resting nucleus apart from the nucleolus. The term chromatin should be applied only to those granules in the resting nucleus that stain with the Feulgen reaction and give rise to chromosomes during mitosis.

The present study of the free-living forms throws light on the probable course of evolution of amoebae. A form like *Naegleria gruberi*, which during division has 'polar masses', 'interzonal bodies' and can readily produce a temporary flagellate stage under certain physiological conditions can be regarded as a primitive amoeba. During the course of evolution it appears that amoebae lose the power to produce 'interzonal bodies', although

* Recently Drs Comandon and de Fonbrune kindly showed me an excellent film on the mitotic division of an amoeba which they assigned to the genus *Acanthamoeba* (Volkonsky 1931), but which in the present classification should be called *Hartmannella*. Comandon & de Fonbrune's (1937) species in some respects resembles *H. rhysodes*. The film clearly shows that the pre-existing chromatin granules in the nucleus move to the centre and give rise to the chromosomes. The nucleolus suddenly disappears before the metaphase stage, and there is no indication that it fragments to give rise to chromosomes as was claimed by Gläser (1912*a*), Dobell (1914) and others. The various stages of nuclear division can be as clearly seen in the living condition of the amoeba in this film as they have been shown in the species of *Hartmannella* described in this work by the use of the Feulgen reaction.

the ability to produce temporary flagella is retained. *Didascalus thorntoni* has been found to produce temporary flagella with difficulty and does not possess 'interzonal bodies' during nuclear division. In the genus *Schizopyrenus* a form like *S. russelli* has been found not to produce a temporary flagellate stage, although the nuclear division of this amoeba is indistinguishable from that of *Didascalus thorntoni*, as are the active forms. These two species of amoebae can be readily distinguished by the characters of their cysts and the process of excystment. Thus it seems that *Schizopyrenus russelli*, *S. erythaenusa* and *S. atopus* have lost their primitive character of temporary flagella production but do retain the family character in having 'polar masses' during nuclear division. When we come to the more advanced family Hartmannellidae, the nucleus, as in *Hartmannella glebae*, *H. rhysodes*, *H. leptocnemus* and *H. agricola* or the nucleoli as in *Pelomyxa carolinensis* (Kudo 1947) and other forms, disappear during division. A spindle with chromosomes arranged as an equatorial plate is formed, as in higher plants and animals. The formation of 'polar masses' during nuclear division and the power to produce temporary flagella under certain conditions are completely lost.

The study of nuclear division, using Feulgen reaction, of the organisms placed in the family Rhizomastigidae would be interesting in establishing the relationship of these organisms to the true amoebae. If it was found that they showed a type of division similar to that found in the family Schizopyrenidae, it would throw light on the phylogenetic relationships between Rhizopoda and Mastigophora.

VI. Suggested classification of amoebae

The classification is based on the nuclear division and possible phylogenetic relationships presented in this work, and on the nuclear structure and modes of nuclear division described by other workers who have used Feulgen reaction successfully. The amoebae fall into two main groups according to the mode of nuclear division. In the first group the nucleolus divides into two, giving rise to 'polar masses'; in the second group the nucleolus or the nucleoli disappear. In both groups the pre-existing chromatin gives rise to chromosomes, and at the end of nuclear division the mass of fused Feulgen-positive chromosomes fragments and returns to the position seen in a resting nucleus.

The amoebae in which the nucleolus persists may be further divided according to whether or not Feulgen-negative material ('interzonal body') collects between the chromosomal plates at anaphase, as shown by Rafalko (1947) and by the present writer in *Naegleria gruberi*; in the amoebae described here the presence of 'interzonal bodies' is associated with the existence of a temporary flagellate stage.

These two distinct types of nuclear division seem to provide a logical basis for dividing the order Amoebida Calkins into two new families, Schizopyrenidae and Hartmannellidae. Such a system of classification throws light on the probable trends of evolution of amoebae.

SCHIZOPYRENIDAE n.fam.

Definition. The resting nucleus contains a more or less central Feulgen-negative nucleolus, which during mitosis divides to form 'polar masses'. 'Interzonal bodies' may be present. Amoebae may have more than one nucleus, and some genera may produce temporary flagella.

The family Schizopyrenidae has been named after the type genus *Schizopyrenus* in accordance with the International Rules of Zoological Nomenclature. At present it is difficult to divide this family into genera but three readily identifiable genera are given below.

TYPE GENUS: *Schizopyrenus*; other genera *Naegleria* and *Didascalus*.

Genus *SCHIZOPYRENUS* n.g.

DEFINITION. Feulgen-negative nucleolus dividing during mitosis to form 'polar masses'. Temporary flagella are not produced.

TYPE SPECIES: *Schizopyrenus russelli* n.sp. Two other species, *S. erythaenusa* n.sp. and *S. autopus* n.sp., are also described.

Schizopyrenus is selected as the type genus because the amoebae included in it do not produce temporary flagella, and it is possible that the presence of temporary flagella may be considered by some authorities to exclude these forms from true amoebae. In any case the production of flagella seems of less importance for diagnostic purposes than the type of nuclear division.

Genus *NAEGLERIA* Alexeieff em. Aut.

DEFINITION. 'Polar masses' are formed. Feulgen-negative 'interzonal bodies' are present during late stages of nuclear division. Temporary flagella are produced.

TYPE SPECIES: *Naegleria gruberi* (Schardinger).

Genus *DIDASCALUS* n.g.

DEFINITION. 'Polar masses' without 'interzonal bodies' are present during nuclear division. Temporary flagella are produced. Type species *Didascalus thorntoni* n.sp.

HARTMANNELLIDAE n.fam.

DEFINITION. This family is created after the well-known type genus *Hartmannella*.

The resting nucleus has either a single Feulgen-negative nucleolus or several nucleoli. During mitosis the nucleolus, or nucleoli, disappear, and a spindle with chromosomes arranged as an equatorial plate resembling that found in higher animals and plants occurs. Amoebae may be uni- or multinucleate, and no temporary flagella have been discovered.

The great majority of free-living and parasitic amoebae seem to belong to this family. No attempt is made at present to give a complete list of genera. The type genus *Hartmannella* is defined and a few other genera are given below merely as an illustration.

Genus *HARTMANNELLA* Alexeieff em. Aut.

DEFINITION. The resting nucleus contains a single Feulgen-negative nucleolus. During mitosis the nucleolus disappears and a spindle with chromosomes arranged as an equatorial plate is formed. No temporary flagella are produced.

TYPE SPECIES: *Hartmannella glebae* (Dobell); and three others, *H. rhysodes* n.sp., *H. leptocnemus* n.sp. and *H. agricola* (Goodey), are dealt with in the foregoing pages.

Alexeieff (1912*b*, *c*) proposed the genus *Hartmannella* for the amoebae characterized by the absence of 'polar masses' during division and the utilization of all or nearly all the chromatic material in the formation of the equatorial plate.

Genus *AMOEBA* Ehrenberg

The exact limits of this genus are difficult to define. It is reasonable to retain the generic name *Amoeba* for some of the large free-living uninucleate forms like *A. proteus*, *A. dubia* and *A. discoides* which have several Feulgen-negative nucleoli in the resting nucleus instead of a single nucleolus. The mitotic division in *A. proteus* (Liesche 1938) is characteristic of the family Hartmannellidae.

Genus *PELOMYXA* Greef

The amoebae belonging to this genus are multinucleate and divide by plasmotomy. The nuclear structure and the mode of nuclear division is similar to that found in *Amoeba proteus*. Kudo (1947) has recently given an excellent account of the nuclear structure and the mode of nuclear division in *P. carolinensis* Wilson by the use of Feulgen reaction.

The position of most of the genera of free-living and of intestinal and parasitic amoebae cannot be determined with certainty till the nuclear division has been fully studied with Feulgen reaction. In the intestinal and some of the parasitic amoebae, judging by the occasional mitotic figures given in various publications, e.g. Calkins (1933) (figures J–4 for *Entamoeba coli*); Wenyon (1926) (two figures on p. 100 showing the division of nuclei in the cysts of *Entamoeba histolytica*), it seems that most of the genera will have to be placed in the family Hartmannellidae.

The recognition of certain genera and species will, however, ultimately depend on careful study of the morphology and biology under standardized and reproducible cultural conditions. Characters such as the form and size of active amoebae under identical cultural conditions, the presence or absence of a well-defined ectoplasm and endoplasm, the method of the movement of the amoebae, characters of cyst, the method of excystment, the division of nuclei inside the cysts and the type of spindle produced during nuclear division, etc., will prove very useful in identifying amoebae. The *limax* character as emphasized in the past, has not much diagnostic value.

The knowledge of nuclear structure and the mode of nuclear division, although essential in creating families and certain genera, will not solve the problem of a more detailed system of classifying amoebae.

Remarks on the earlier systems of classifying amoebae included in the family Schizopyrenidae

In the past the presence of temporary flagella in amoebae has been regarded as of sufficient importance to create the family Dimastigamoebidae Wenyon or Bistadiidae Doflein. Since the work of Vahlkampf (1905) amoebae possessing 'polar masses' during nuclear division have been classified on the presence or absence of temporary flagella. Chatton & Lalung-Bonnaire (1912) created the genus *Vahlkampfia* for amoebae possessing 'polar masses' during nuclear division and pores in the cyst wall. No flagellate forms were observed, but the conditions necessary for the production of flagella were not tested.

Alexeieff (1912*c*) proposed the genus *Naegleria* for amoebae showing 'polar masses' during division. As some forms belonging to this genus showed flagellate stage, Calkins (1913) assigned the genus *Vahlkampfia* to those not producing temporary flagella and *Naegleria* to those possessing both amoeboid and flagellate stages. Thus the amoebae characterized by 'polar masses' and the presence of temporary flagella have been included in the family Dimastigamoebidae and in the genera *Amoeba*, *Naegleria*, *Vahlkampfia* and others in the family Amoebidae Bronn. Those without flagella have been put in the genera *Amoeba*, *Vahlkampfia* and in some others. It is impossible to tell from most of the earlier publications, owing to the incomplete descriptions of nuclear division, whether the amoebae, possessing 'polar masses' with or without flagella, had 'interzonal bodies'. The earlier literature on classification is in such a confused state that a detailed review here would not serve any useful purpose. However, a few remarks justifying the recognition of three distinct genera, *Schizopyrenus*, *Naegleria* and *Didascalus*, in the family Schizopyrenidae may have some value.

The creation of a family Dimastigamoebidae is not justified. It seems more logical to put species such as *Didascalus thorntoni* and *Schizopyrenus russelli*, described in this work, whose nuclear divisions are indistinguishable, in the same family rather than to follow Wenyon (1926), Calkins (1933), Kudo (1946) and others in placing them in different families merely on the ground that one can occasionally produce temporary flagella while the other, as far as is known, cannot. The character of temporary flagella production then becomes of generic value only.

For the first time emphasis is laid, in the present classification, on the presence or absence of 'interzonal bodies' as a diagnostic character in recognizing the genera *Naegleria* and *Didascalus* both of which produce temporary flagella. Judging from the presence of 'interzonal bodies', it seems that Vahlkampf's (1905) *limax* amoeba was a species of *Naegleria*, although no temporary flagella were recorded by him. His measurements of the amoeba (0·75 by 1·5 to 4·0μ with nuclei 0·3 to 0·5μ in diameter and the cysts 1·5μ in diameter) should be multiplied by 8 or 10 times as pointed out by Alexeieff (1911). Several instances from the earlier literature can be cited in which an amoeba possessing 'interzonal bodies' during nuclear division has been found to possess temporary flagella when the necessary conditions were provided. Gläser (1912*a*), in *N. tachypodia*, figured distinct 'interzonal bodies' but did not record the presence of a temporary flagellate stage. When this amoeba was reinvestigated by Pietschmann (1929), she had no difficulty in finding the temporary flagella, although she wrongly placed the organism in the genus *Vahlkampfia*. Wenyon's (1926) *Dimastigamoeba gruberi* (see his figure 5, p. 105) seems to possess distinct 'interzonal bodies', although these structures, when stained with iron-alum haematoxylin, were interpreted as aggregated daughter chromosomes passing towards the two poles. If, however, an amoeba were found which did not possess a temporary flagellate stage and did produce 'polar masses' and 'interzonal bodies' during nuclear division, the genus *Vahlkampfia* would have to be recognized, and *V. limax* would become the type species. At present the existence of such a genus is considered doubtful. The possession of pores in the cyst wall, which was suggested by Chatton & Lalung-Bonnaire (1912) as one of the diagnostic characters of the genus *Vahlkampfia*, suggests that they were dealing with a species of *Naegleria*.

It is difficult to assign the genus *Sappinia* Dangeard to the family Schizopyrenidae with certainty till the nuclear division has been studied carefully by Feulgen reaction in a form like *S. diploidea* (Hartmann & Nägler). Nägler's (1909) cytological work on *S. diploidea* is rather poor, although it suggests that this amoeba may possess 'polar masses' during nuclear division. Looking at one or two late stages in the nuclear division of this amoeba, kindly shown to me by Miss Crump from her iron-alum haematoxylin preparations made many years ago, I could not make up my mind if 'polar masses' were really present.

Bunting (1922, 1926) and Bunting & Wenrich (1929) described in the flagellate *Tetramitus rostratus* Perty, obtained in cultures made from the caecal contents of rat, an interesting life cycle consisting of amoeboid and flagellate phases. According to them the organism encysts only in the amoeboid phase and the cysts give rise to amoebae after excystation. More recently this organism has been studied by Hollande (1937, 1942). He has created a new family Vahlkampfidae to include two genera *Vahlkampfia* and *Tetramitus*. The nuclear division in *Tetramitus rostratus* both in the flagellate and amoeboid phases are of the type described in the family Schizopyrenidae in this work. It is not possible to determine by inspection of the division figures given by Bunting & Wenrich (1929) and Hollande (1942) whether there is a definite 'interzonal body', as described above, in the case of *Naegleria gruberi*; but very recently Rafalko (1951) has given a good account of the mitotic division in this organism using Feulgen reaction, and has shown the presence of an 'interzonal body'. According to the mode of nuclear division it seems perhaps more logical to put the genus *Tetramitus* in the family Schizopyrenidae than to create a new family, as has been done by Hollande (1937, 1942).

VII. Summary

1. A systematic study of nine species of small free-living amoebae has been made, under standardized and reproducible cultural conditions, by a new method that enables specimens in all stages of division to be obtained easily.

2. In all species studied the resting nucleus shows a Feulgen-negative nucleolus and Feulgen-positive chromatin granules.

3. Nuclear division in these species and in other amoebae described by other workers is of two main types on which it is proposed to create two new families, Schizopyrenidae and Hartmannellidae.

4. In the Schizopyrenidae the nucleolus persists throughout division. Within this family the type genus *Schizopyrenus* and two other genera are at present defined.

(1) *Naegleria*, distinguished by the formation of a Feulgen-negative 'interzonal body' between the chromosomal plates during anaphase. In this genus there is a temporary flagellate stage. The type species, *N. gruberi*, is described.

(2) *Didascalus* n.g., in which no 'interzonal body' is formed but which also has a flagellate stage. The type species, *D. thorntoni* n.sp., is described.

(3) *Schizopyrenus* n.g., differing from *Didascalus* in the absence of a flagellate stage. The type species, *S. russelli* n.sp. and two other species *S. erythaenusa* n.sp. and *S. atopus* n.sp., are described.

5. In the family Hartmannellidae, the nucleolus or the nucleoli disappear during nuclear division and are later re-formed. In the type genus *Hartmannella* the nucleus

contains only a single nucleolus. The type species *H. glebae* (Dobell) and three other species, *H. rhysodes* n.sp., *H. leptocnemus* n.sp. and *H. agricola* (Goodey), are described.

6. The relation of the proposed classification to previously defined families and genera of amoebae and its bearing on phylogeny are discussed.

This work was made possible by a grant from the Agricultural Research Council to whom the author's thanks are due. It is a pleasure to express my sincere thanks to Dr H. G. Thornton, F.R.S., for his interest in this work, and to Dr T. Goodey, F.R.S., for his help and advice on nomenclature. I am very grateful to Miss L. M. Crump for her constructive criticisms generously offered throughout the course of this work and for giving me an interesting species of soil amoeba. My thanks are also due to Mr D. A. Boalch for kindly suggesting suitable names for amoebae, Professor E. G. Pringsheim for giving me three strains of *Naegleria gruberi*, and to Miss Mabel Dunkley for valuable assistance in connexion with the preparation of the manuscript.

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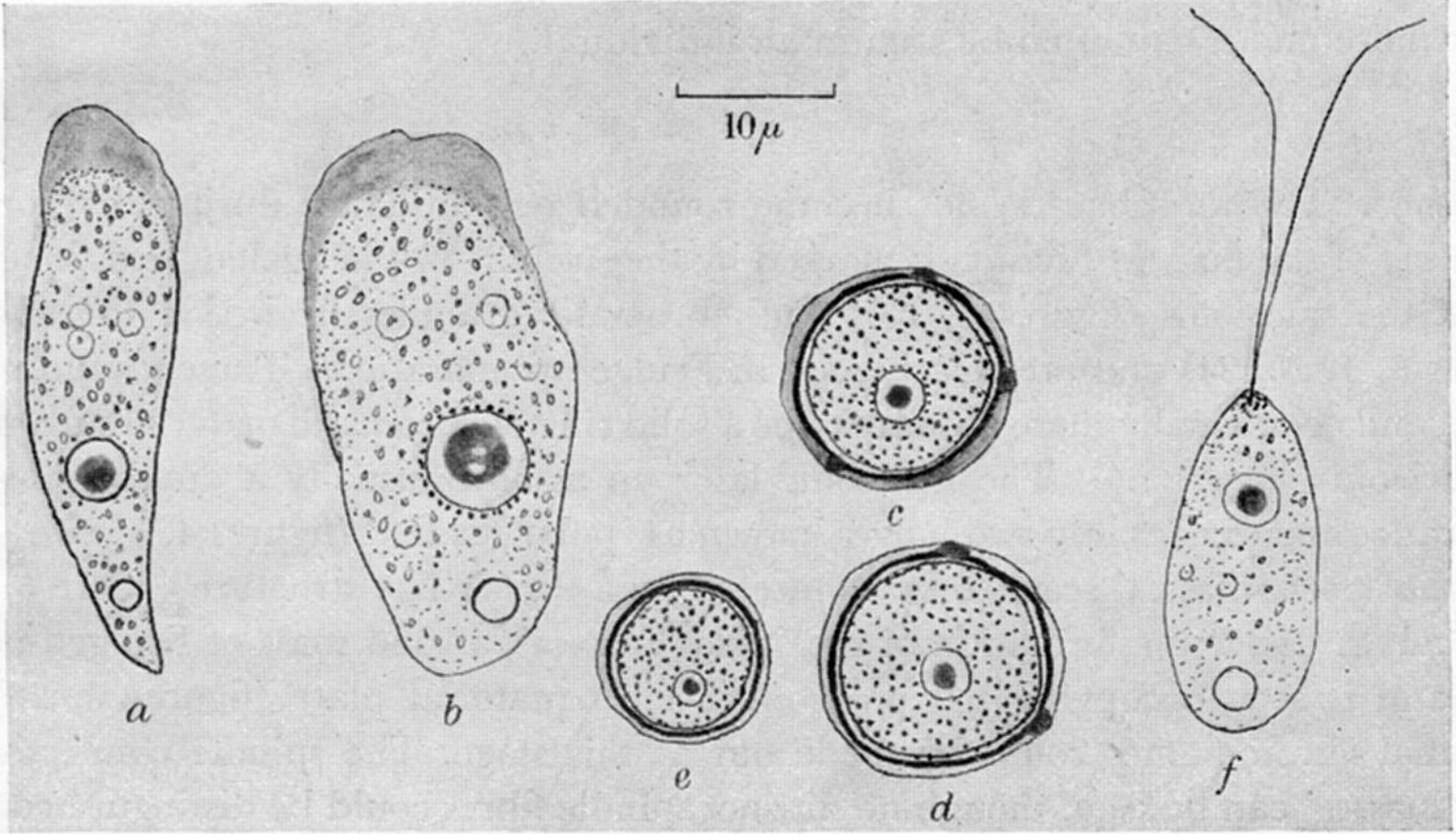
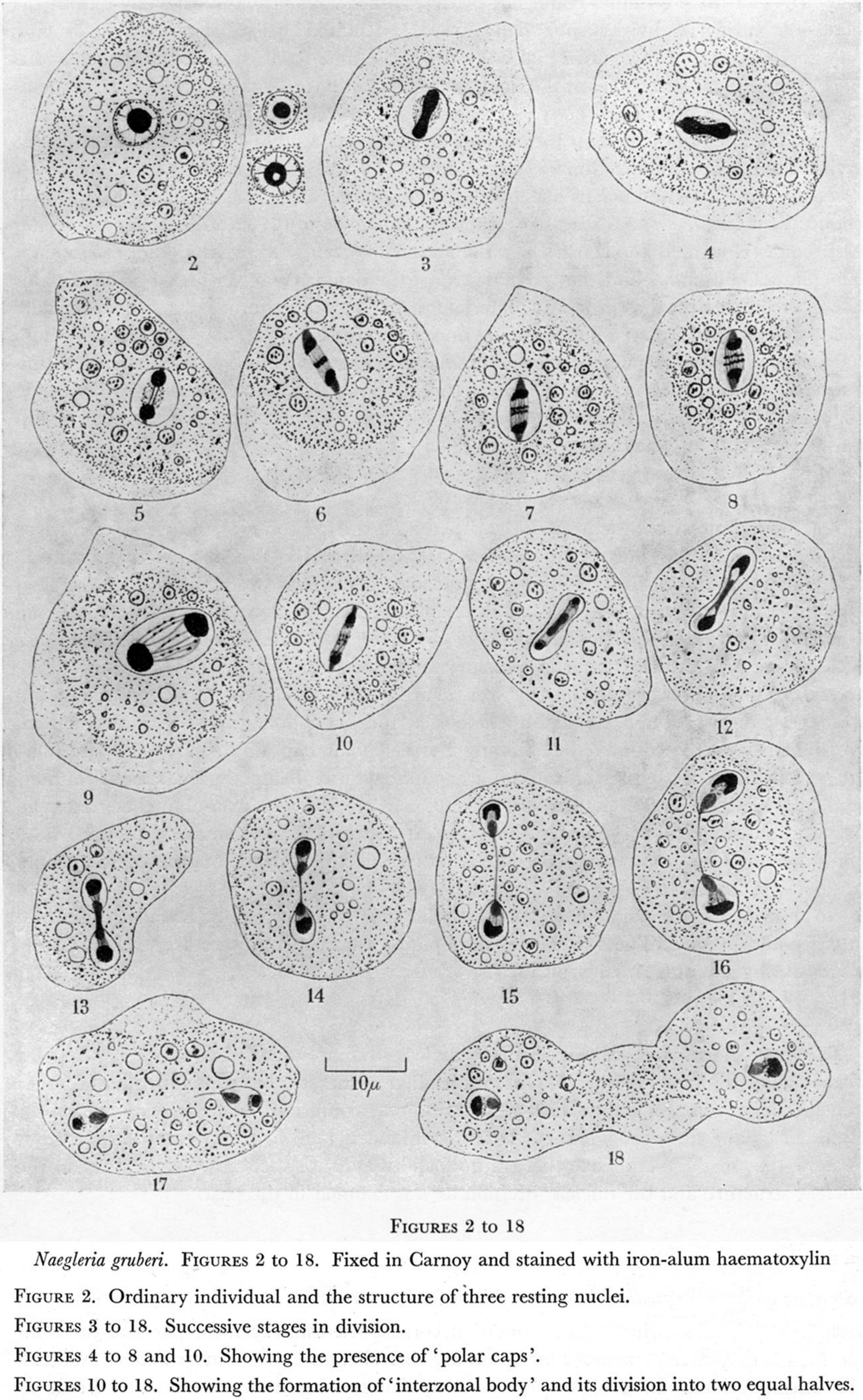


FIGURE 1. *Naegleria gruberi*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, *e*, cysts; *f*, flagellate stage.



FIGURES 2 to 18

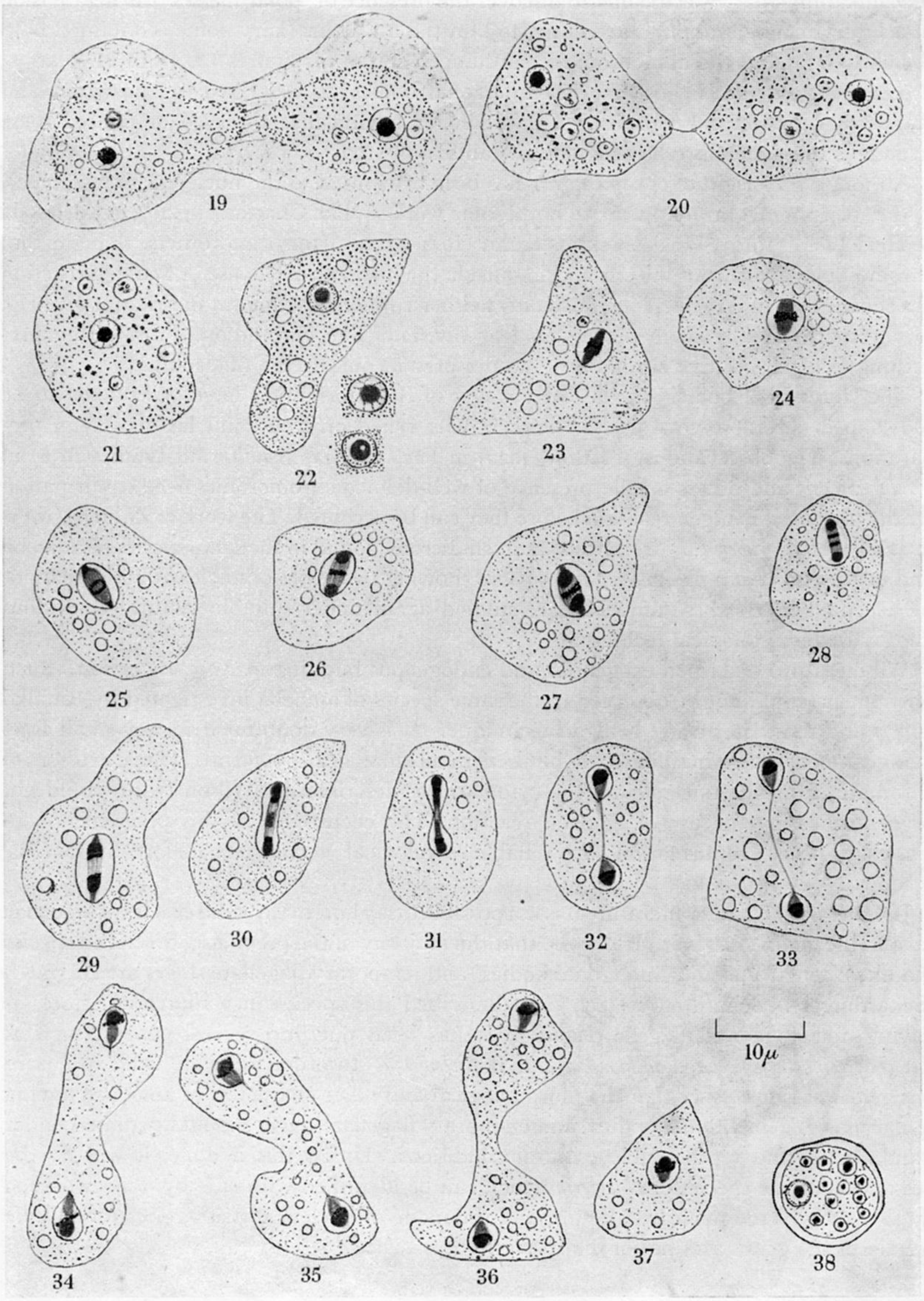
Naegleria gruberi. FIGURES 2 to 18. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 2. Ordinary individual and the structure of three resting nuclei.

FIGURES 3 to 18. Successive stages in division.

FIGURES 4 to 8 and 10. Showing the presence of 'polar caps'.

FIGURES 10 to 18. Showing the formation of 'interzonal body' and its division into two equal halves.



FIGURES 19 to 38

Naegleria gruberi. FIGURES 19 to 21. Fixed in Carnoy and stained with iron-alum haematoxylin and FIGURES 22 to 38 fixed in Carnoy and stained with Feulgen reaction and light green

FIGURES 19 and 20. Successive stages in division.

FIGURE 21. An amoeba just after division.

FIGURE 22. Ordinary individual and three resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 23 to 36. Successive stages in division showing the behaviour of chromatin and the nucleolus.

FIGURES 25, 27 and 29. Showing 'polar caps'.

FIGURE 30. Showing the non-chromatic 'interzonal body' after the chromosomes have moved to the poles and are lying in contact with the 'polar masses'.

FIGURES 31 to 36. Showing the 'interzonal bodies'.

FIGURE 37. Showing the fusion of the 'interzonal body' and the 'polar mass' to give rise to the nucleolus in an amoeba just divided.

FIGURE 38. A cyst showing Feulgen-negative nucleolus and Feulgen-positive chromatin granules in the nucleus.

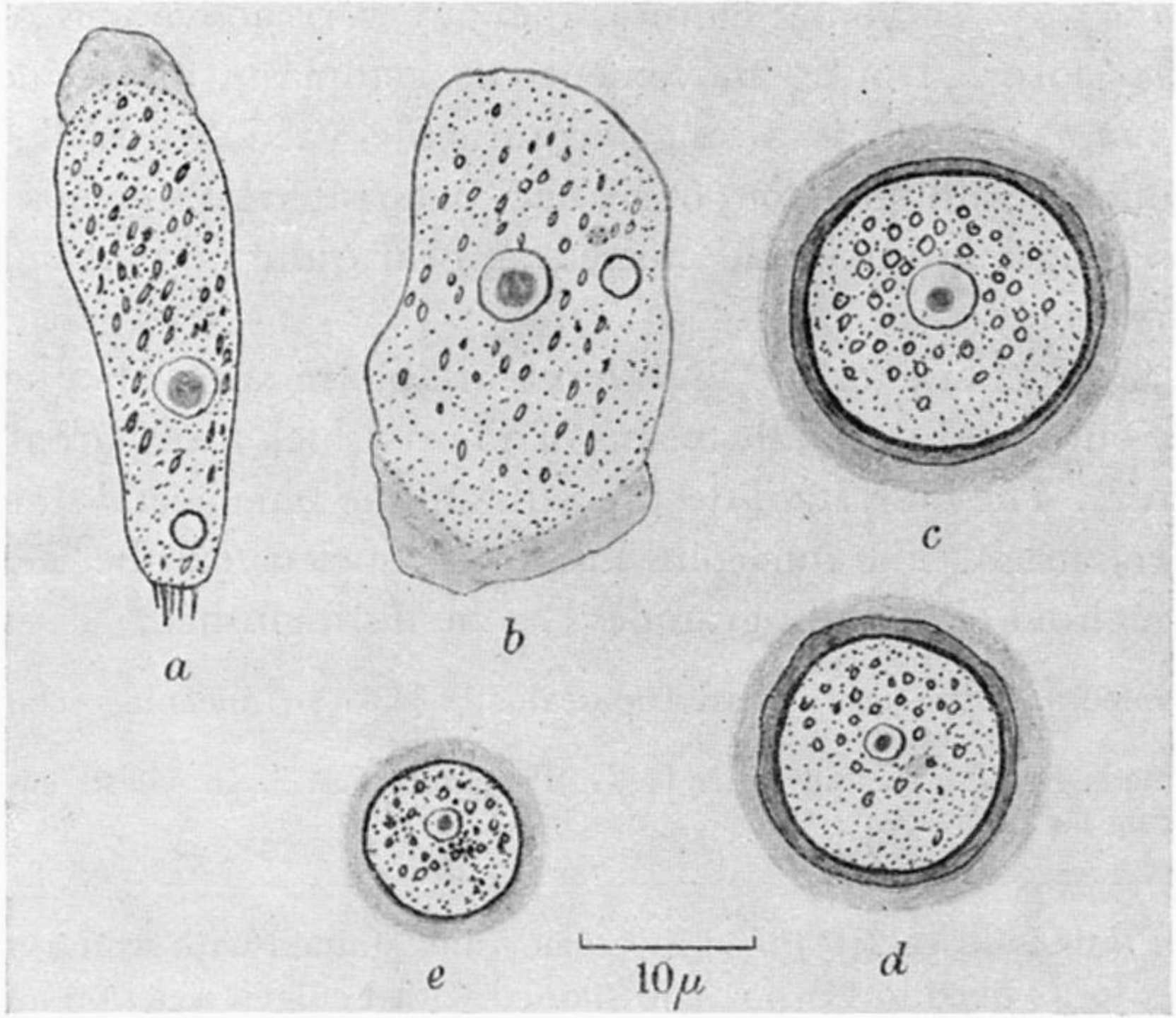
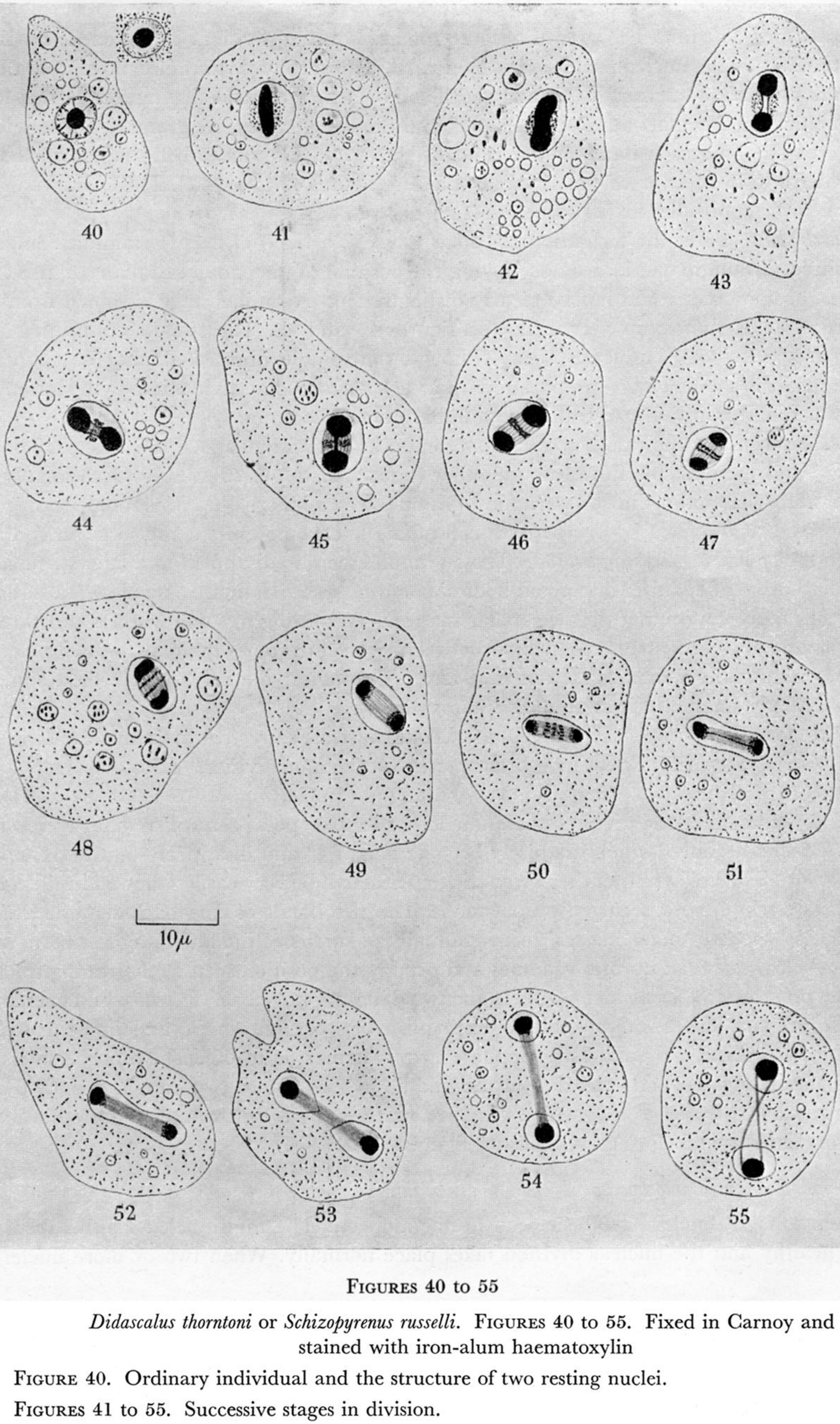


FIGURE 39. *Didascalus thorntoni*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, *e*, cysts.

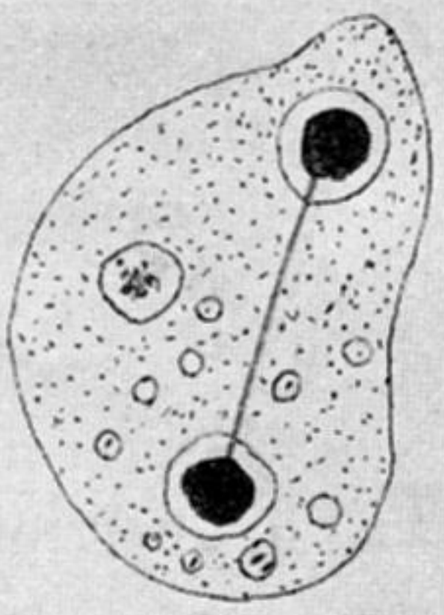


FIGURES 40 to 55

Didascalus thorntoni or *Schizopyrenus russelli*. FIGURES 40 to 55. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 40. Ordinary individual and the structure of two resting nuclei.

FIGURES 41 to 55. Successive stages in division.



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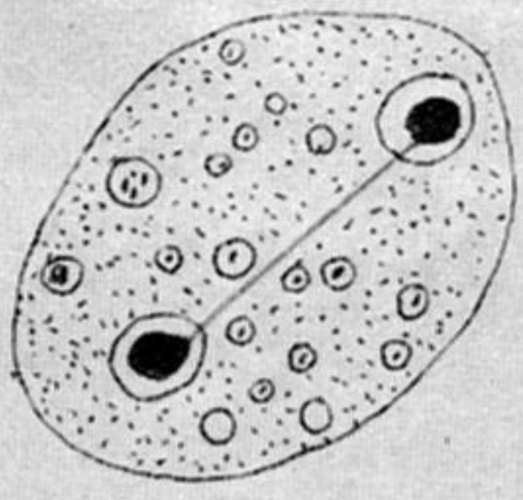

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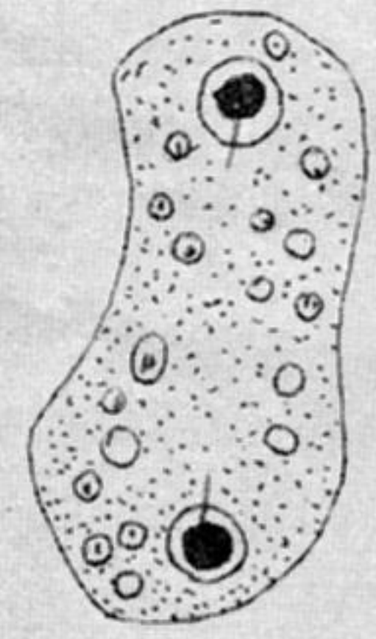

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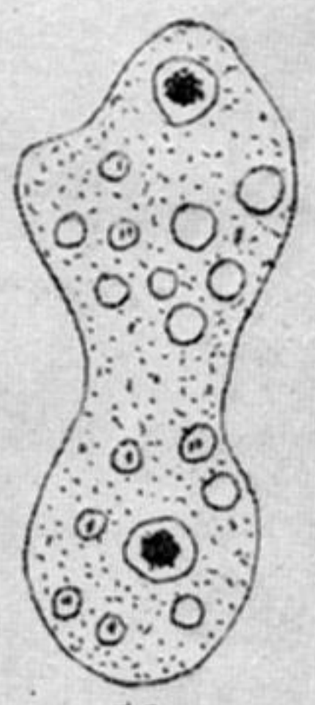

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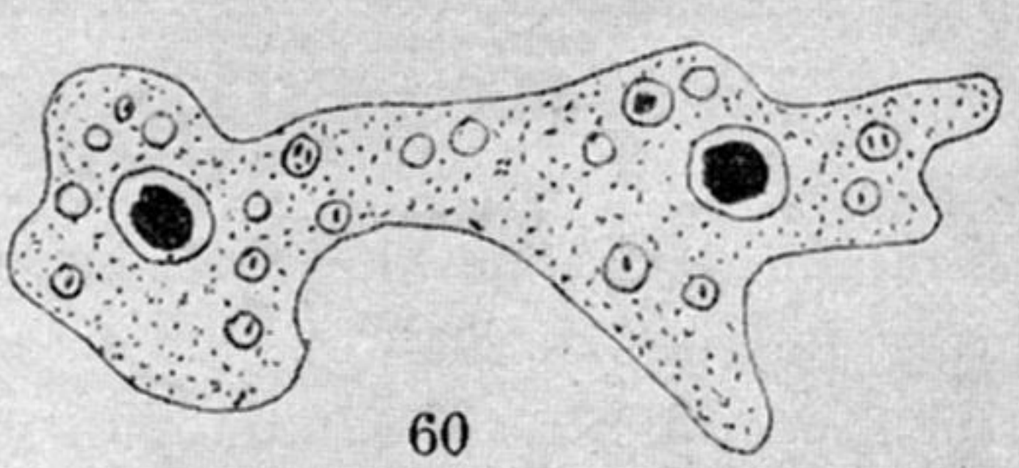

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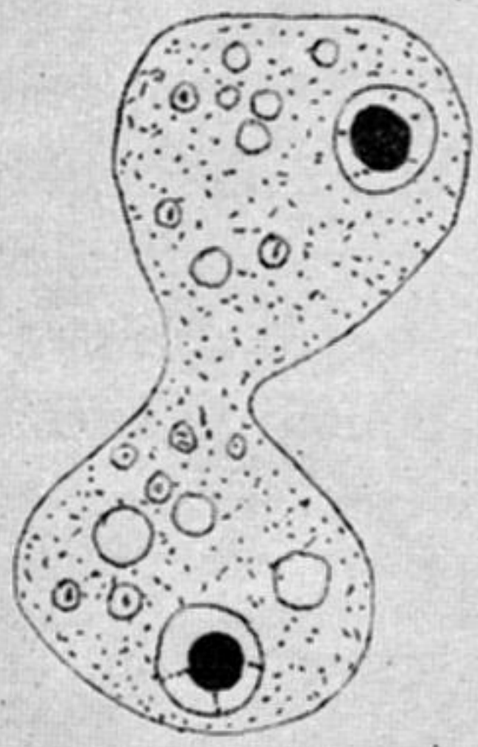

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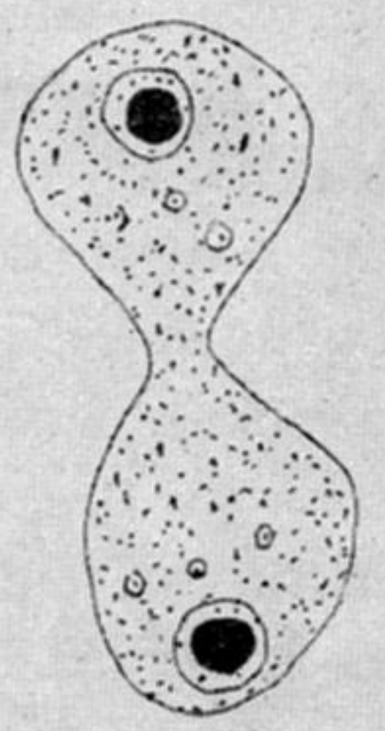

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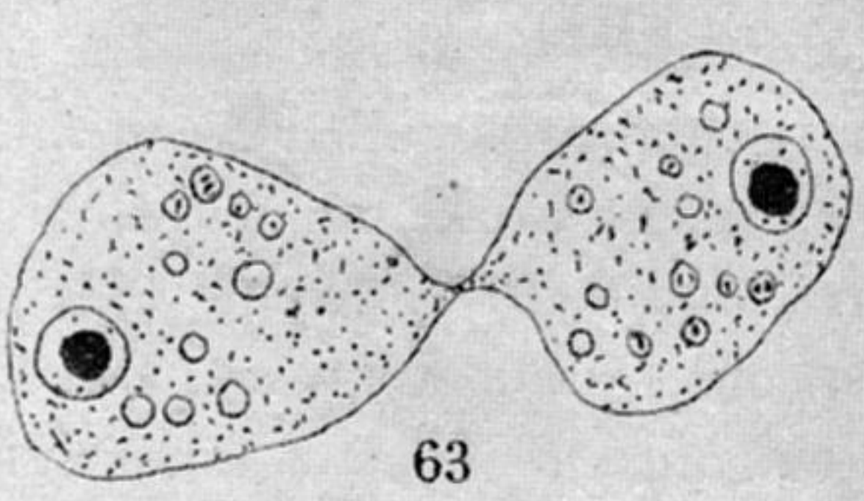

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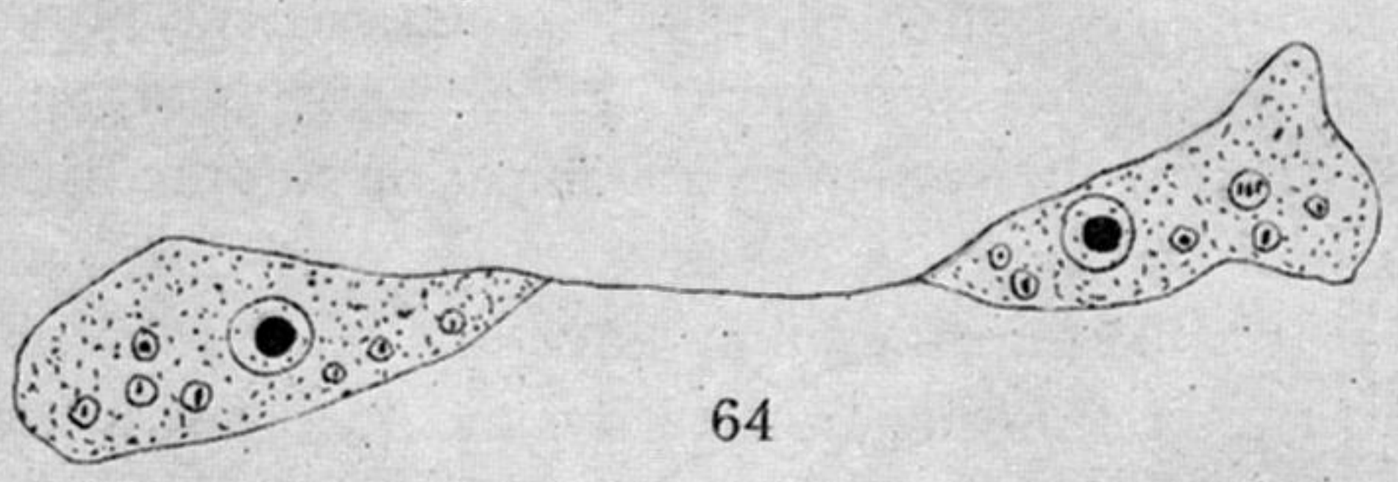

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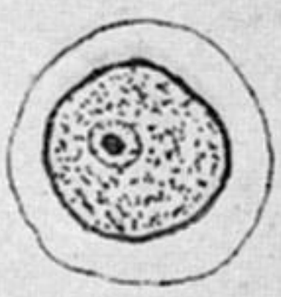

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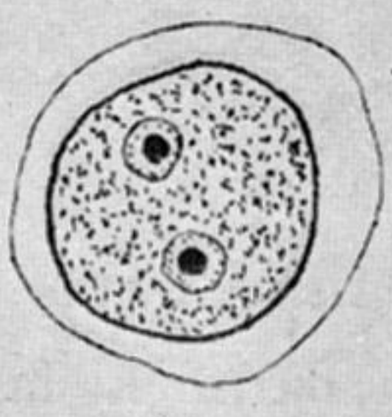

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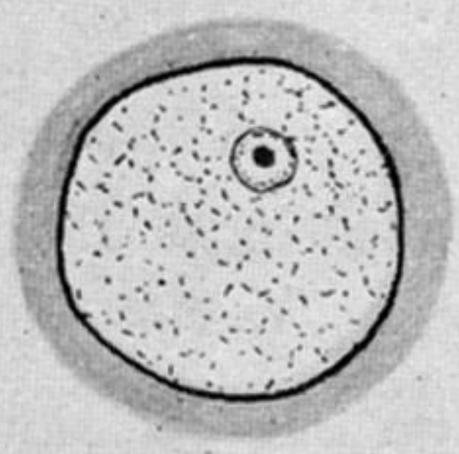

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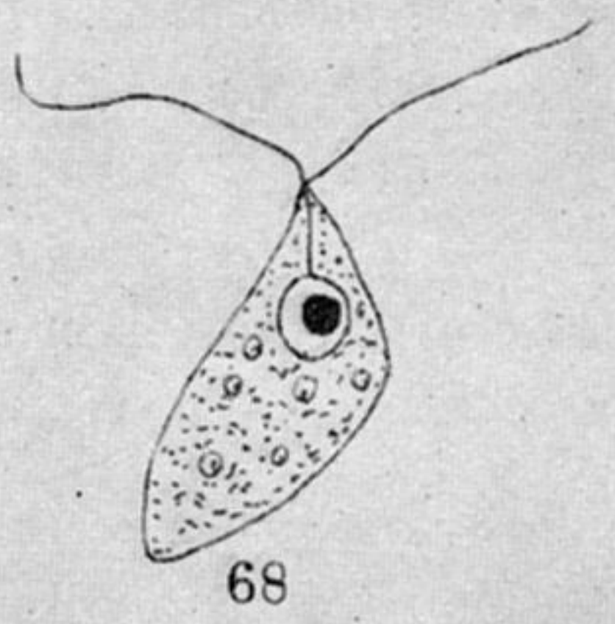

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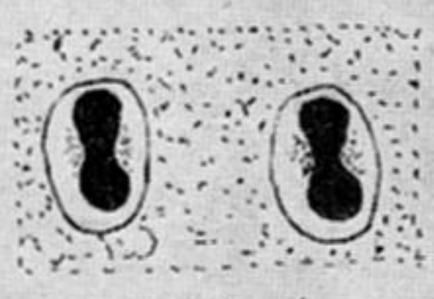

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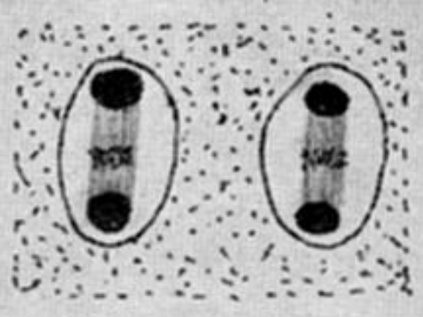

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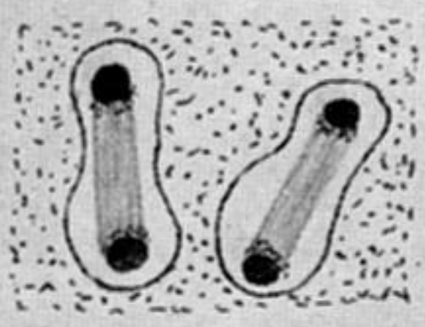

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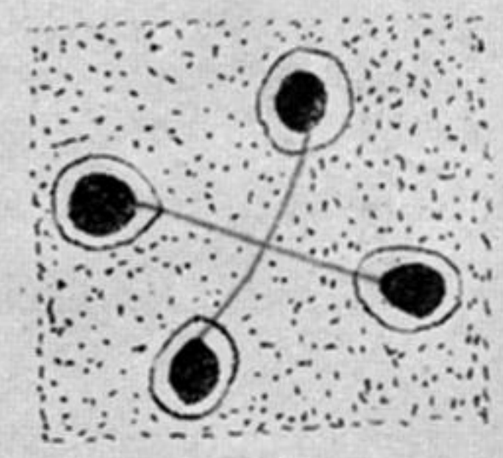

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Figures 56 to 72

Didascalus thorntoni or *Schizopyrenus russelli*

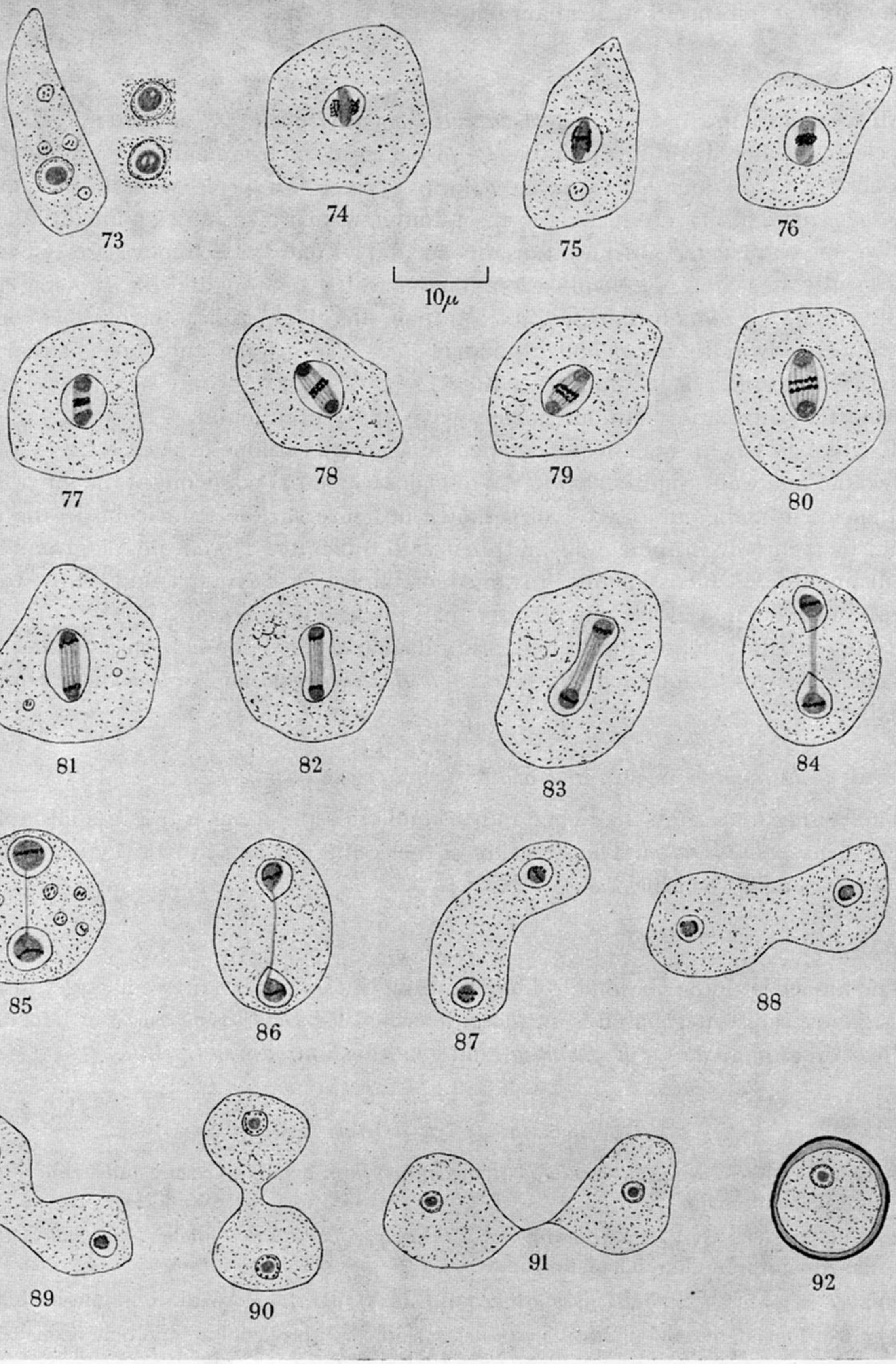
Figures 56 to 64. Showing the successive stages in division. Fixed in Carnoy and stained with iron-alum haematoxylin.

Figures 65 and 66. Cysts of *Schizopyrenus russelli* having one and two nuclei. Fixed in Carnoy and stained with iron-alum haematoxylin.

Figure 67. Cyst of *Didascalus thorntoni*. Fixed in Carnoy and stained with iron-alum haematoxylin.

Figure 68. Flagellate stage of *Didascalus thorntoni* stained with phenol aniline blue acetic acid.

Figures 69 to 72. Stages of nuclear division in *Didascalus thorntoni* having two nuclei. Fixed in Carnoy and stained with iron-alum haematoxylin.



FIGURES 73 to 92

Didascalus thorntoni or *Schizopyrenus russelli*. FIGURES 73 to 92. Fixed in Carnoy and stained with Feulgen reaction and light green

FIGURE 73. Ordinary individual and three resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 74 to 91. Successive stages in division showing the behaviour of chromatin and the nucleolus.

FIGURE 92. Cyst of *Schizopyrenus russelli* showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus.

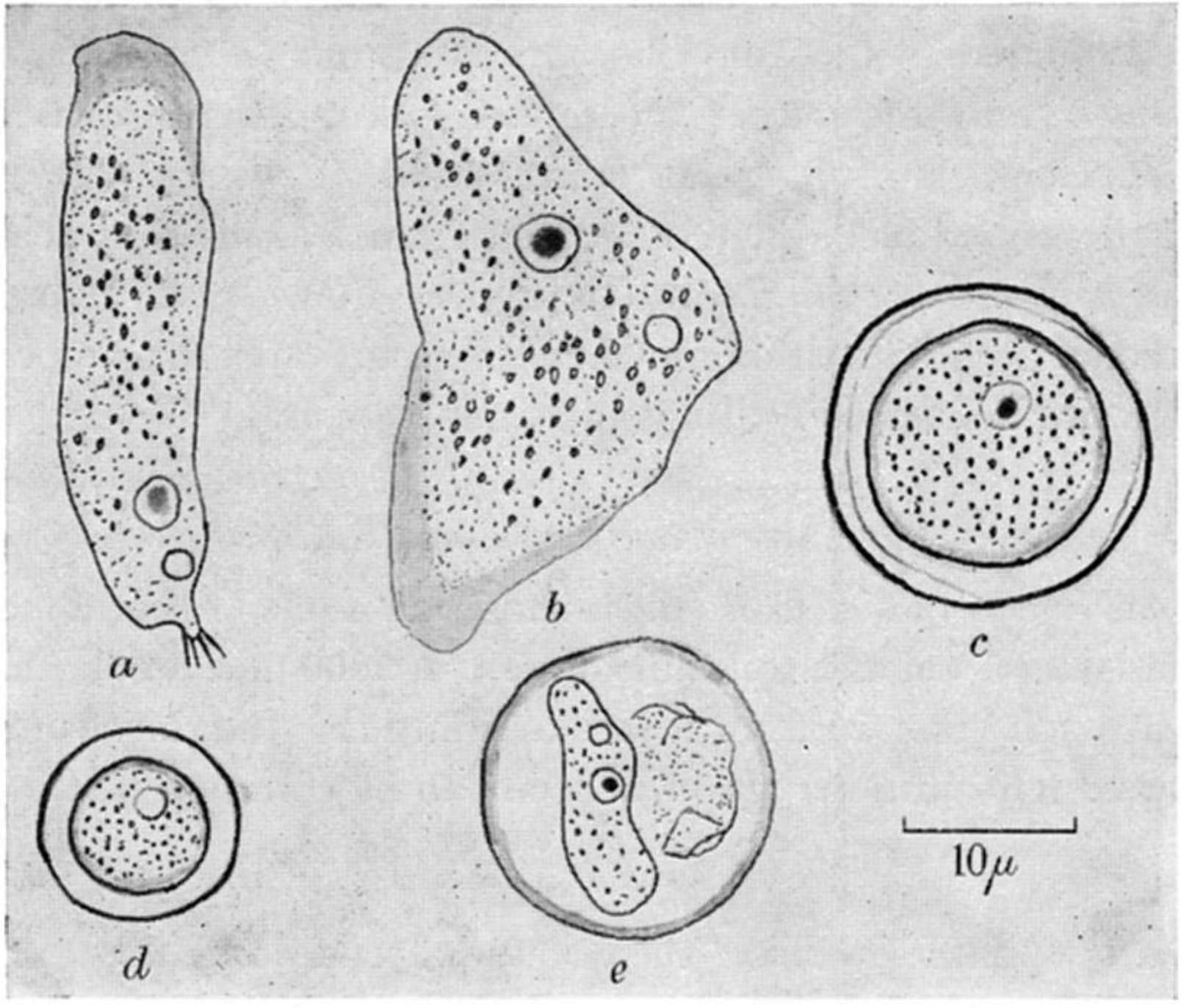


FIGURE 93. *Schizopyrenus russelli*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts; *e*, an amoeba just come out of the inner cyst and still enclosed by the outer cyst wall.

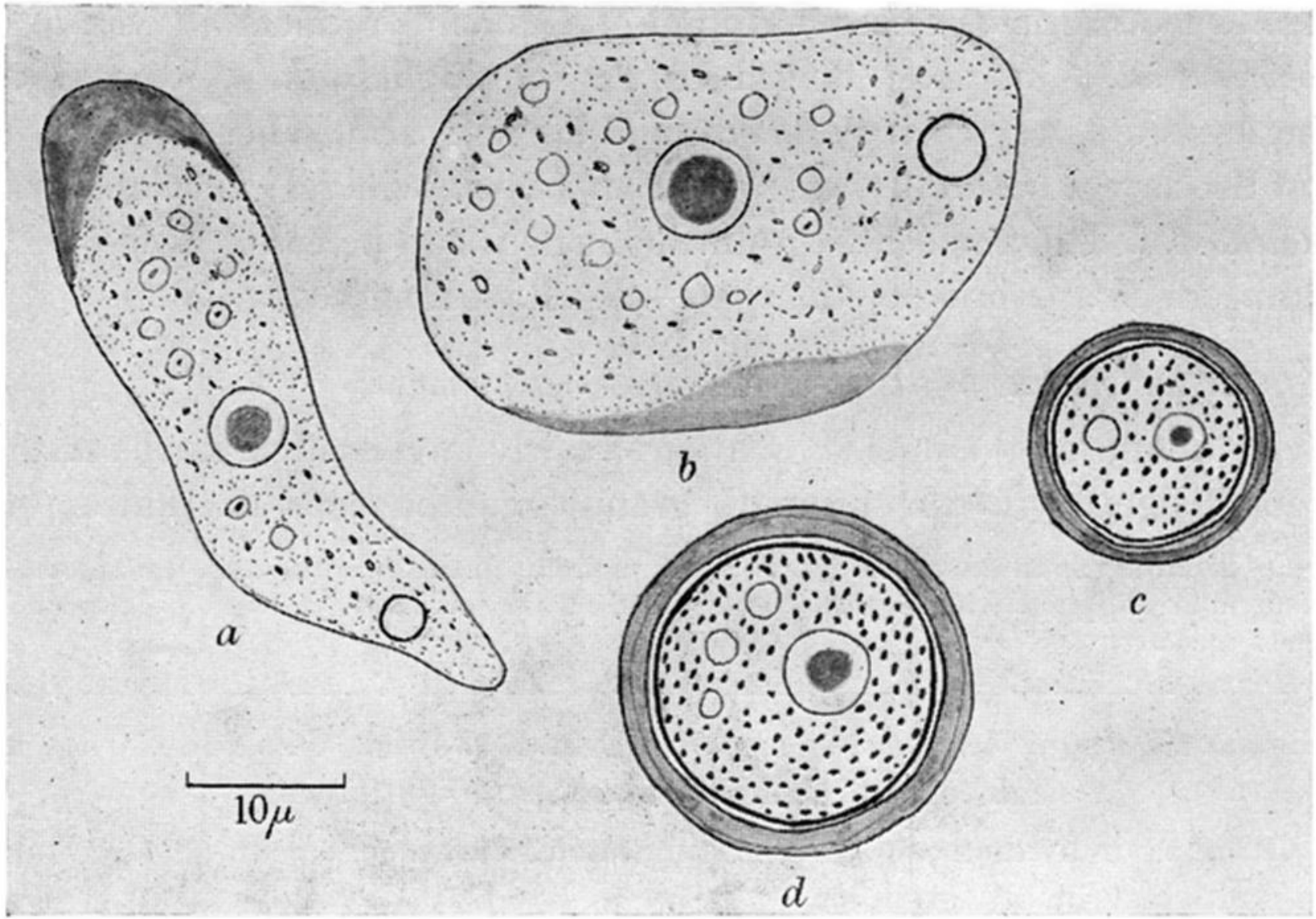
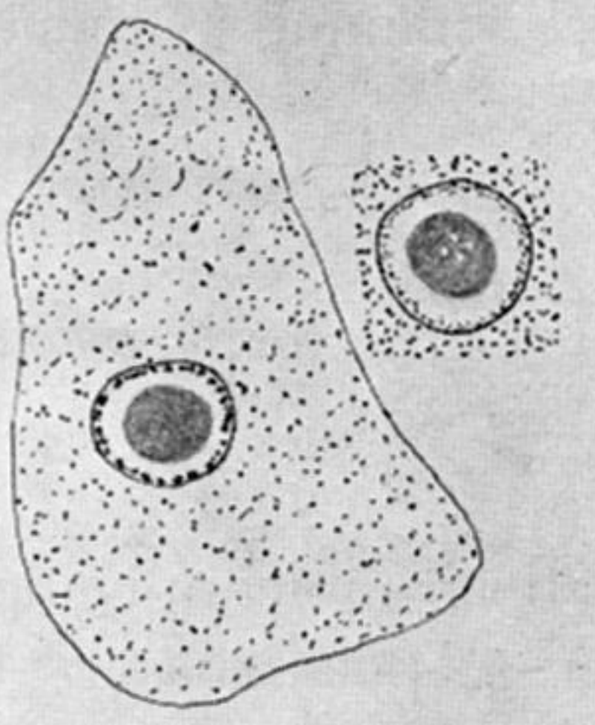


FIGURE 94. *Schizopyrenus erythaenusa*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts.



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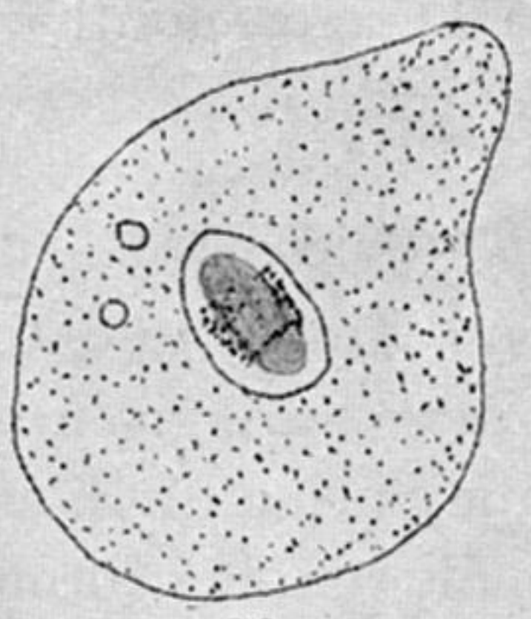

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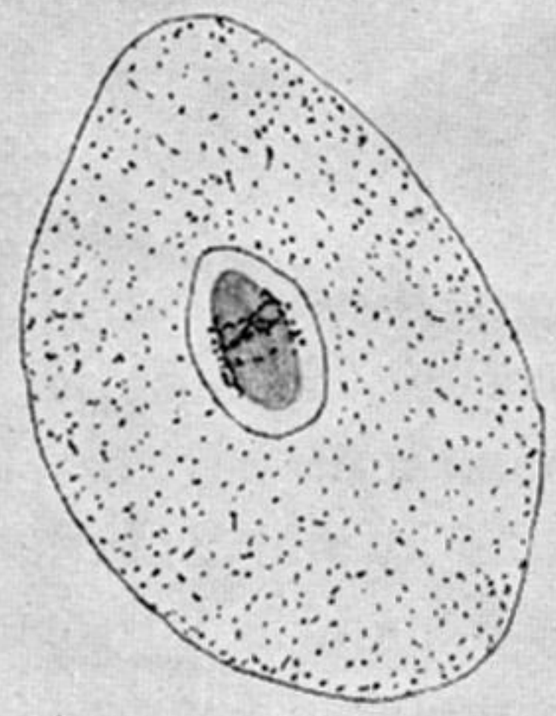

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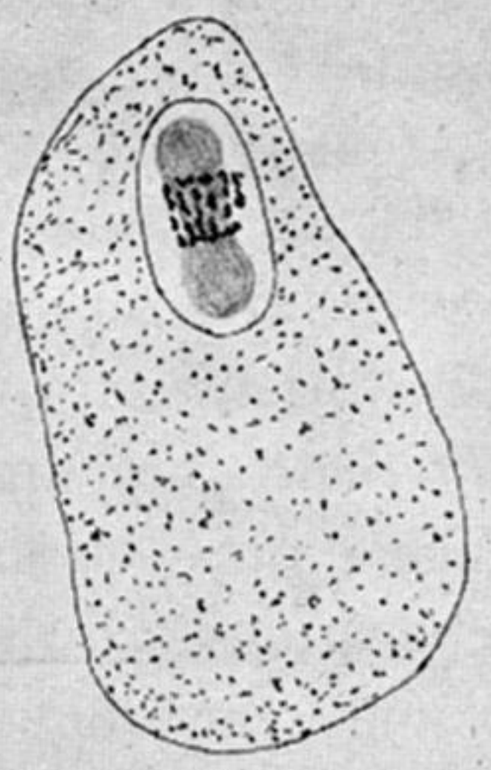

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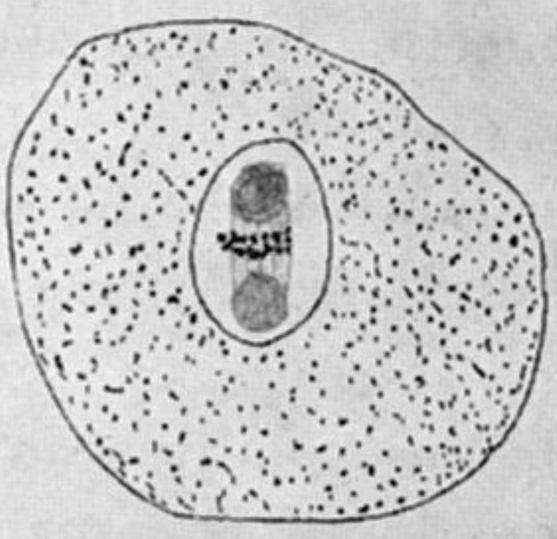
10μ



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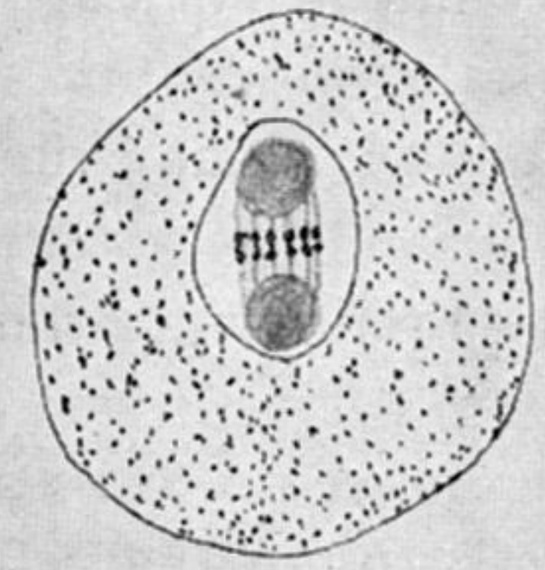

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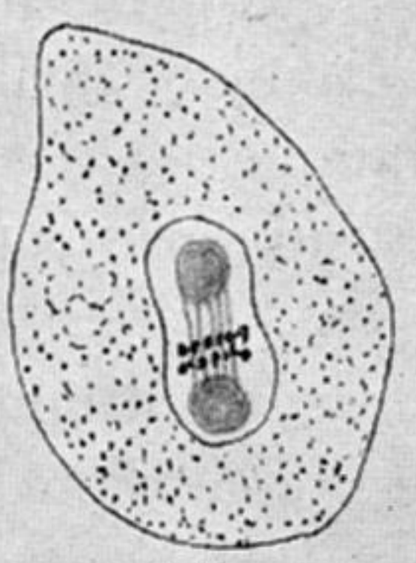

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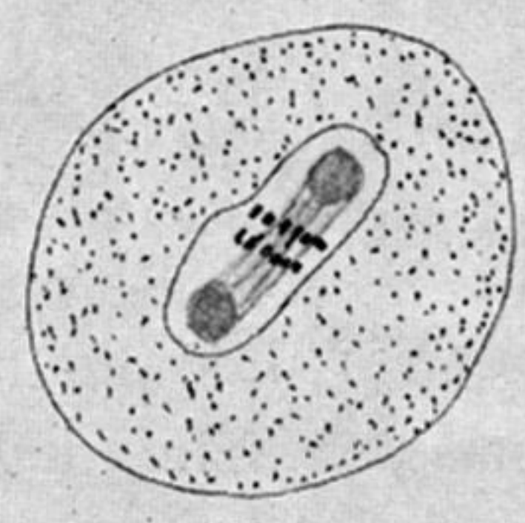

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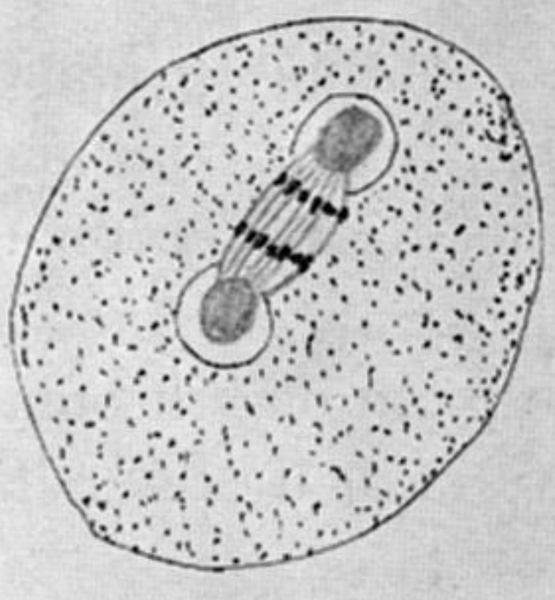

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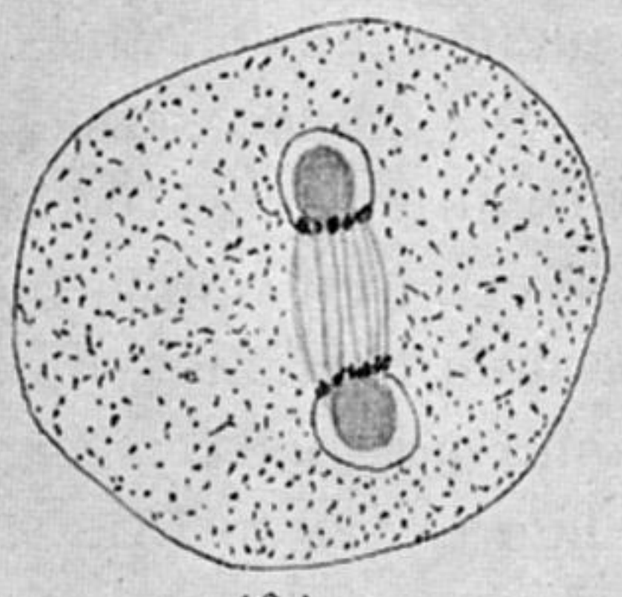

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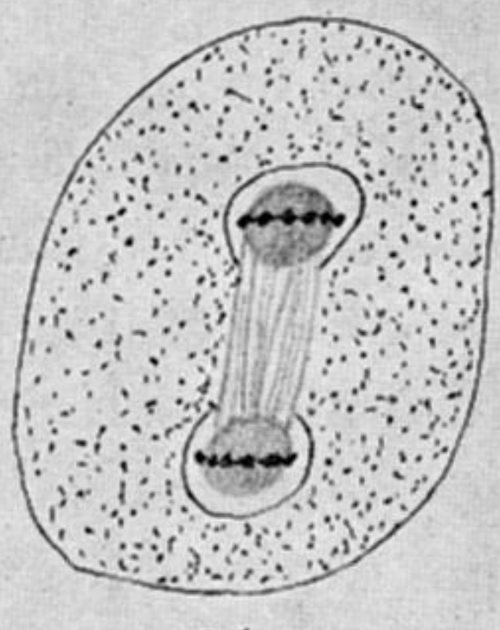

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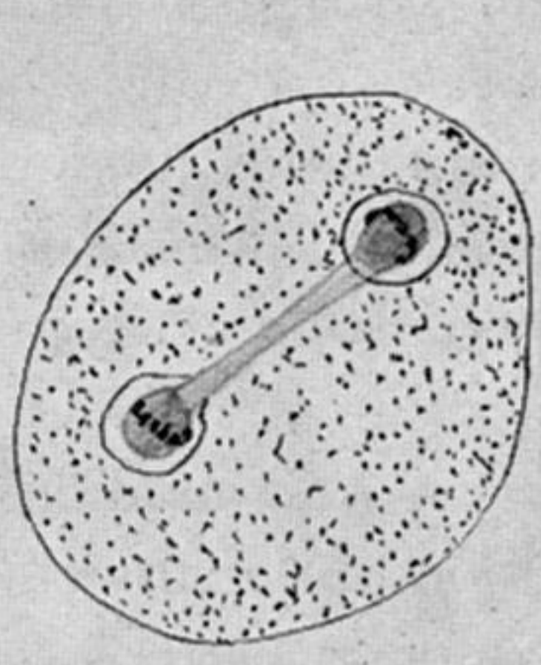

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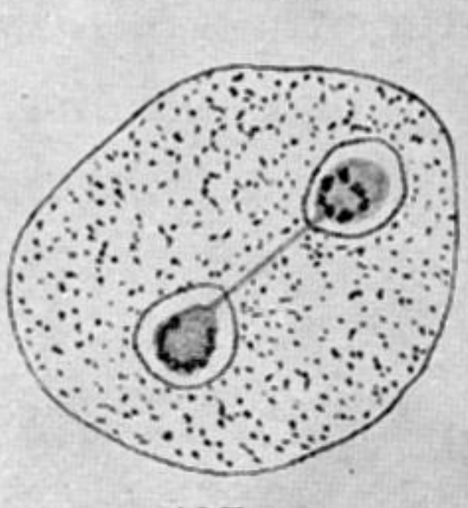

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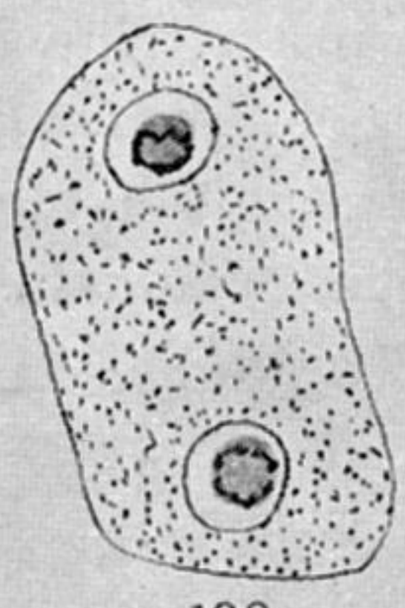

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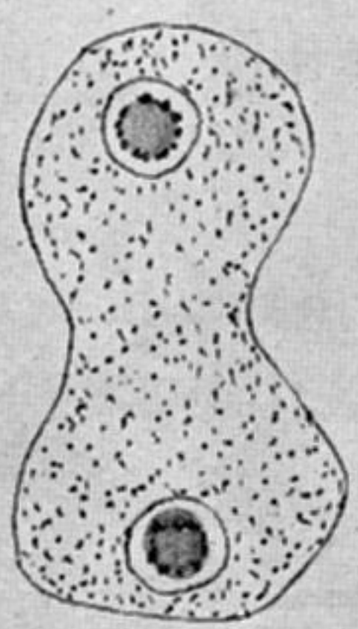

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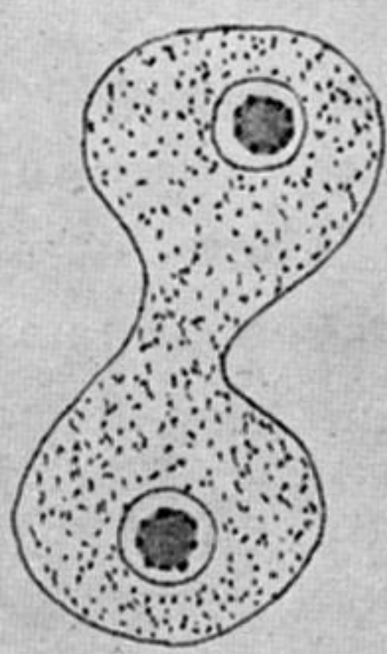


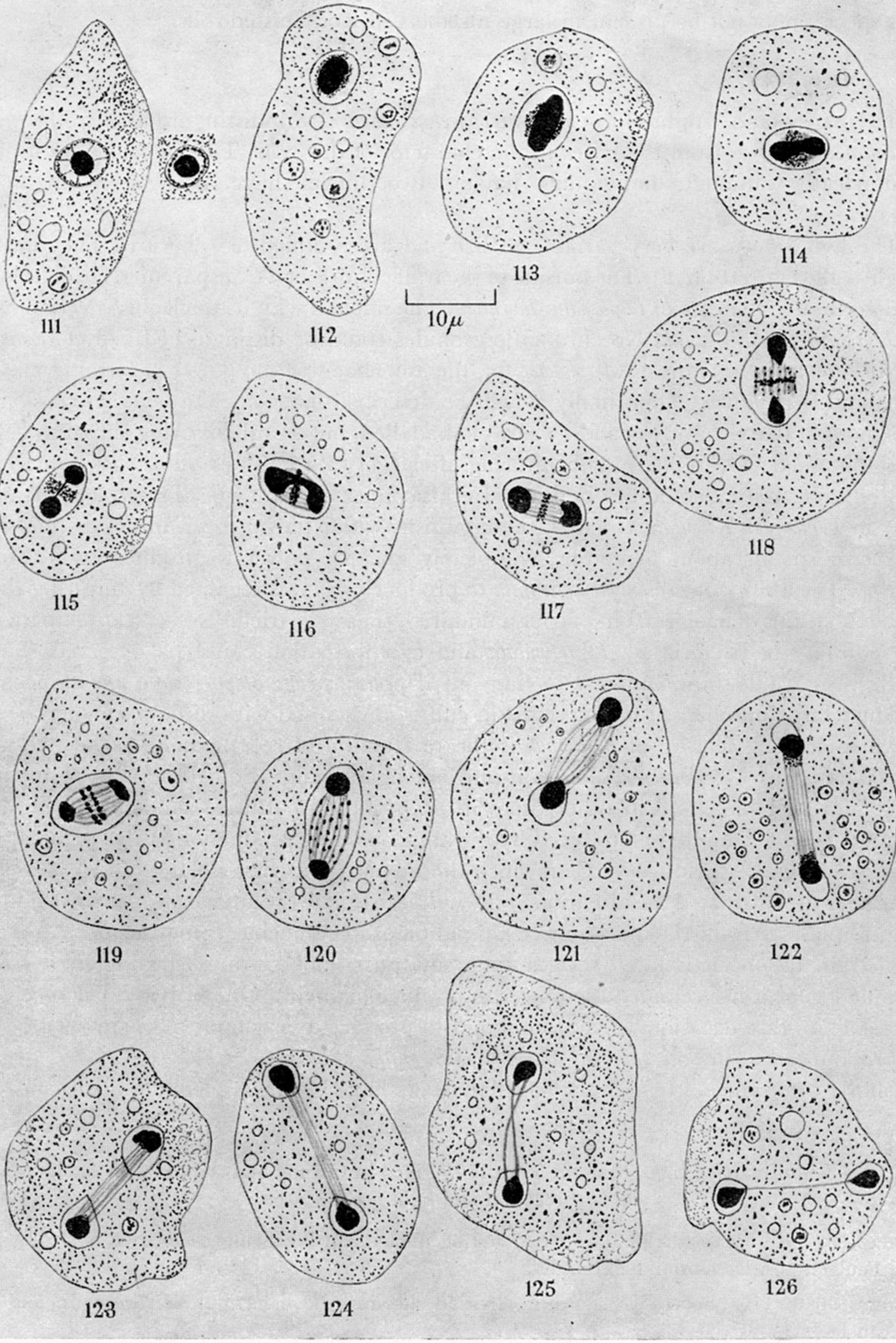
110

FIGURES 95 to 110

Schizopyrenus erythaenusa. FIGURES 95 to 110. Fixed in Carnoy and stained with Feulgen reaction and light green

FIGURE 95. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 96 to 110. Successive stages in division showing the behaviour of chromatin and the nucleolus.

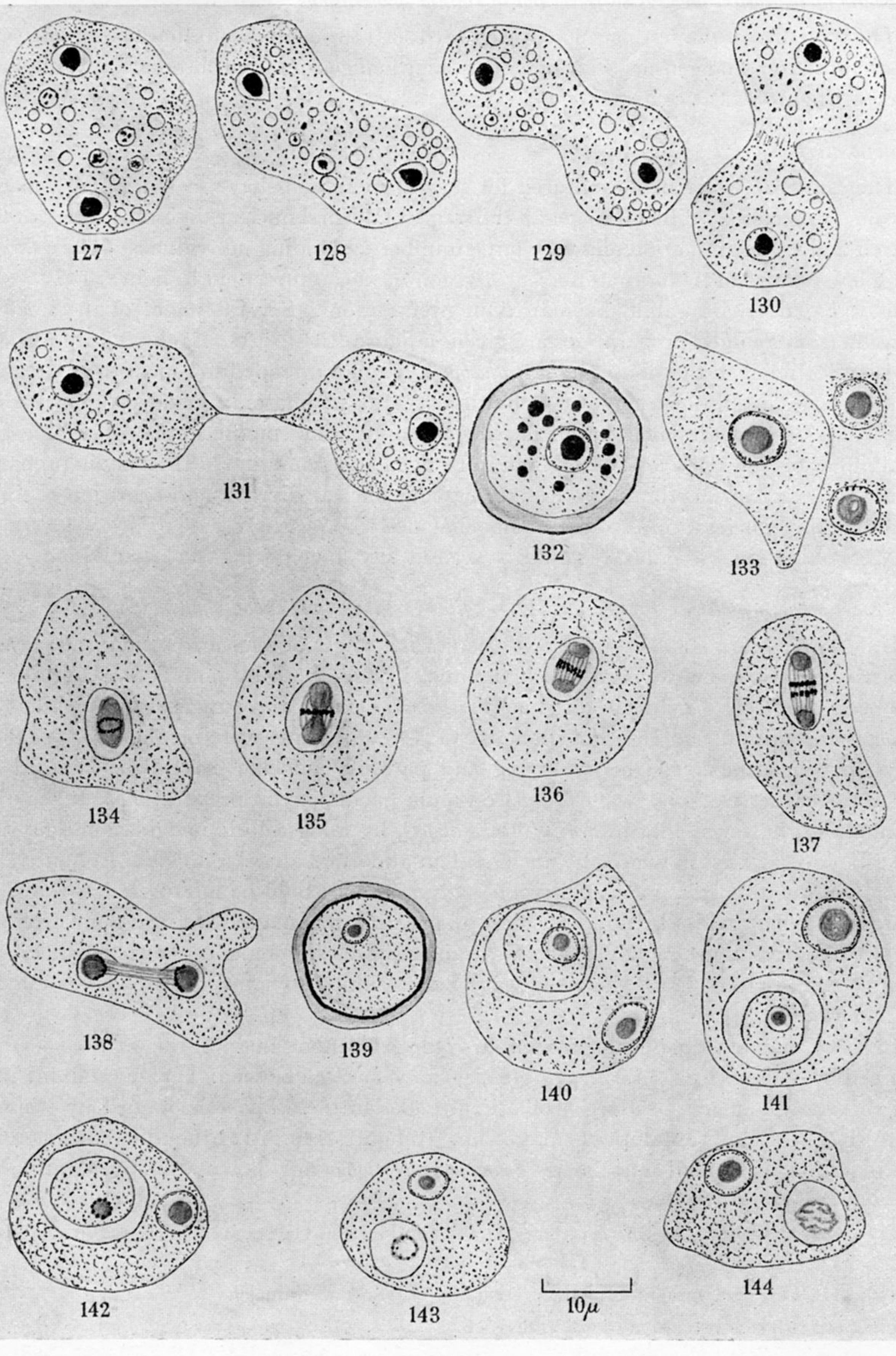


FIGURES 111 to 126

Schizopyrenus erythaenusa. FIGURES 111 to 126. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 111. Ordinary individual and the structure of two resting nuclei.

FIGURES 112 to 126. Successive stages in division.



FIGURES 127 to 144

Schizopyrenus erythaenusa

FIGURES 127 to 131. Stages in division. Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURE 132. A cyst of *Schizopyrenus erythaenusa* showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus. Fixed in Carnoy and stained with Feulgen reaction and light green.

FIGURES 133 to 144. *Schizopyrenus atopus*. Fixed in Carnoy and stained with Feulgen reaction and light green.

FIGURE 133. Ordinary individual and three resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 134 to 138. Stages in division showing the behaviour of chromatin granules and the nucleolus.

FIGURE 139. A cyst showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus.

FIGURES 140 to 144. Stages in the digestion of a small amoeba resembling endogenous bud formation.

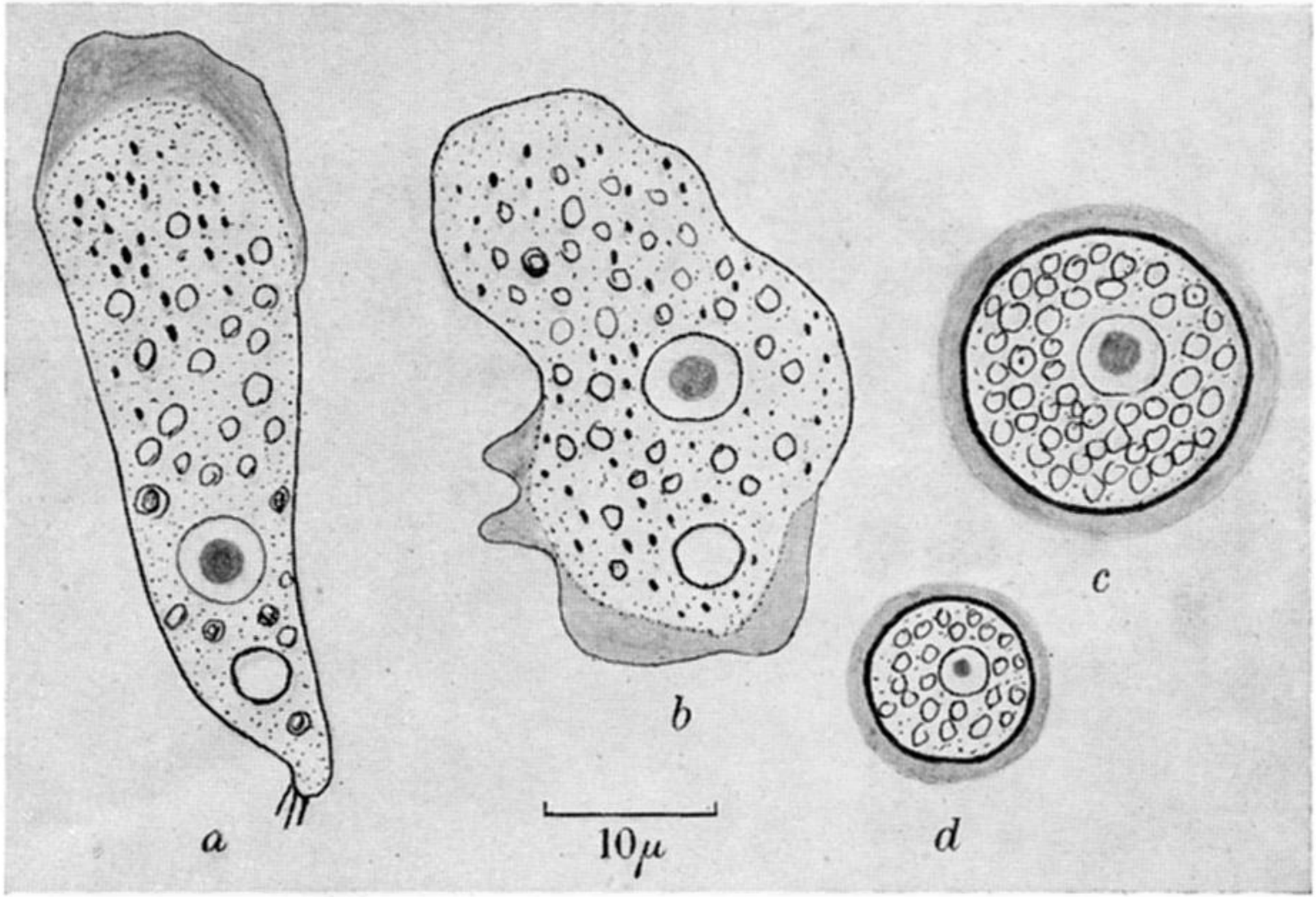
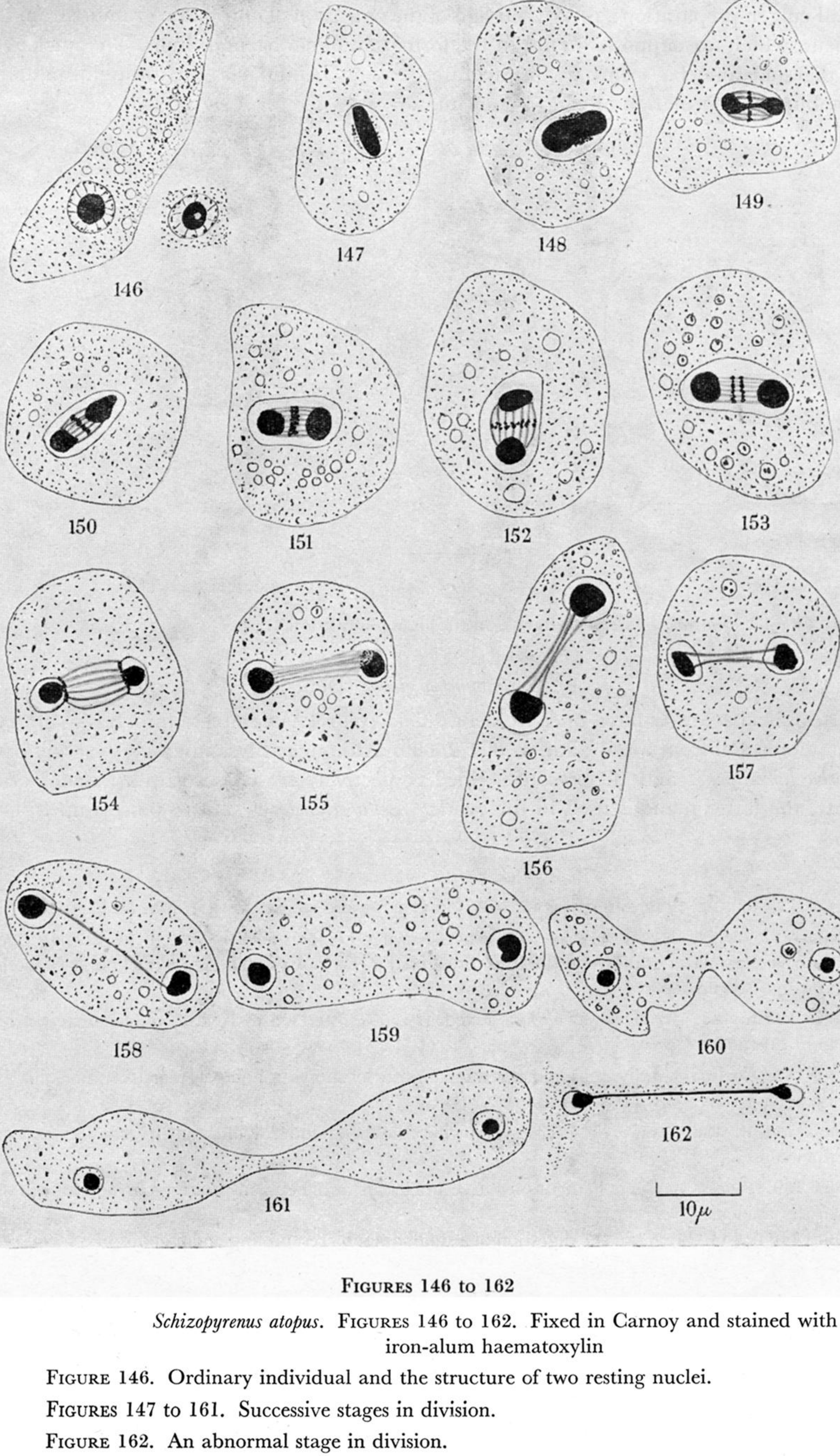


FIGURE 145. *Schizopyrenus atopus*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts.



FIGURES 146 to 162

Schizopyrenus atopus. FIGURES 146 to 162. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 146. Ordinary individual and the structure of two resting nuclei.

FIGURES 147 to 161. Successive stages in division.

FIGURE 162. An abnormal stage in division.

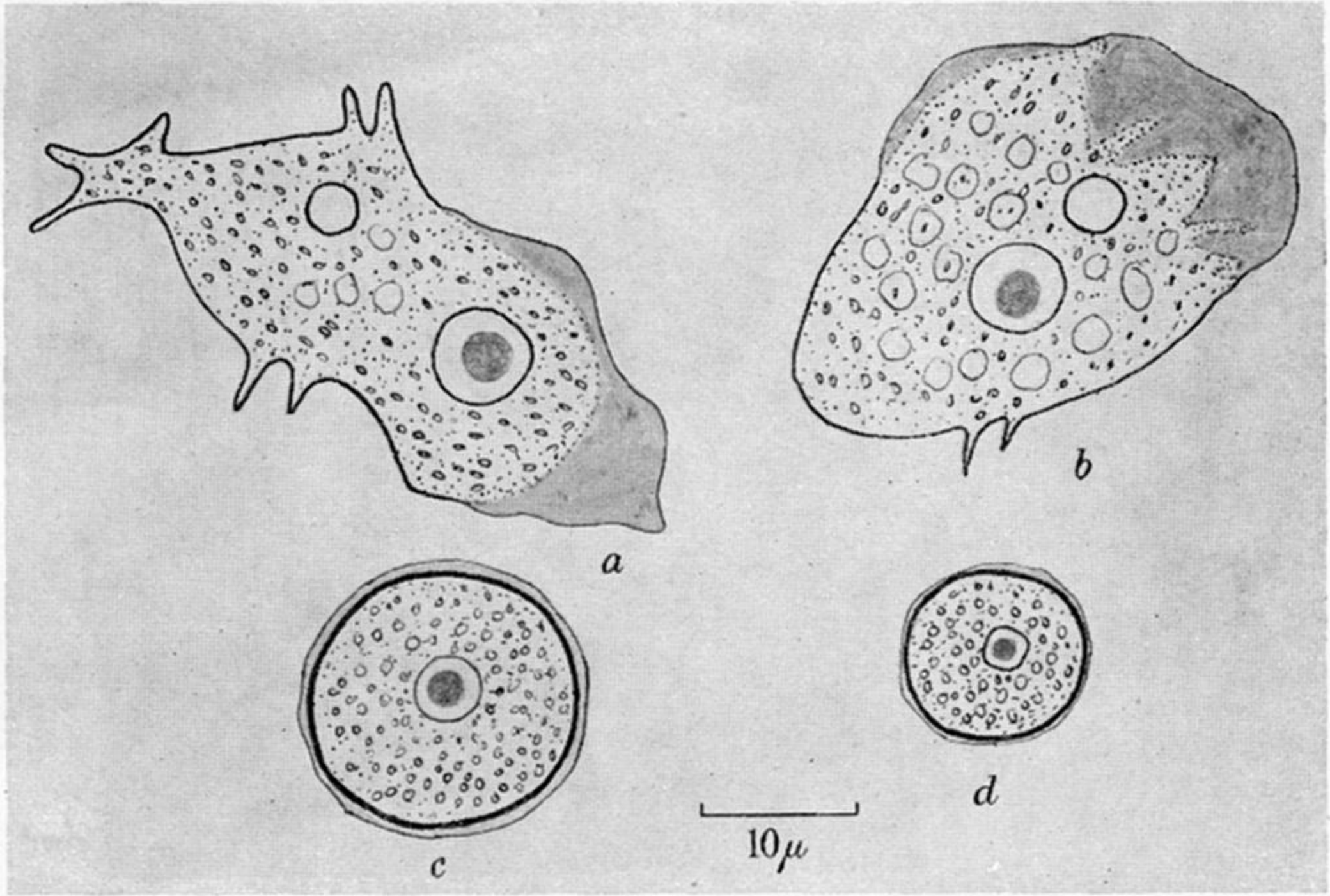
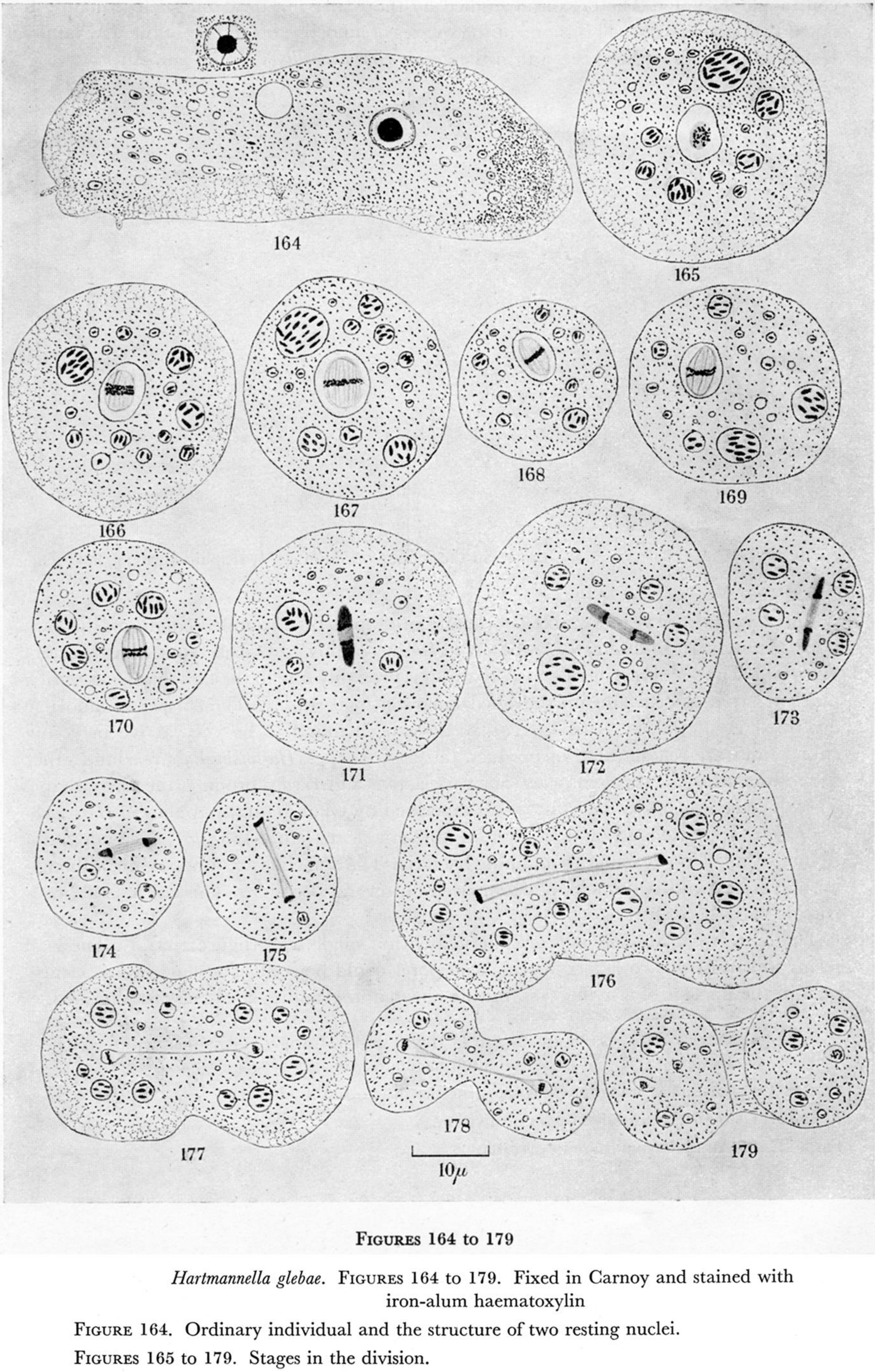


FIGURE 163. *Hartmannella glebae*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, cysts.

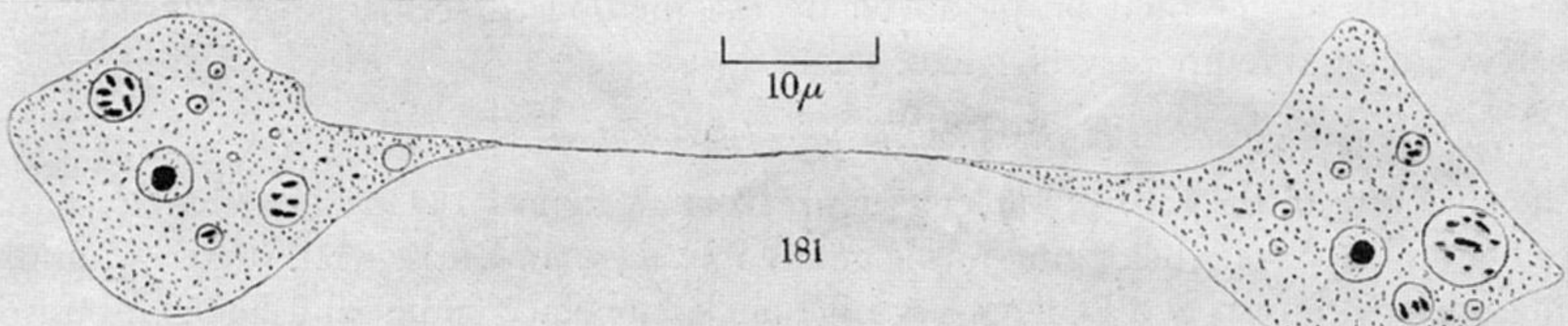


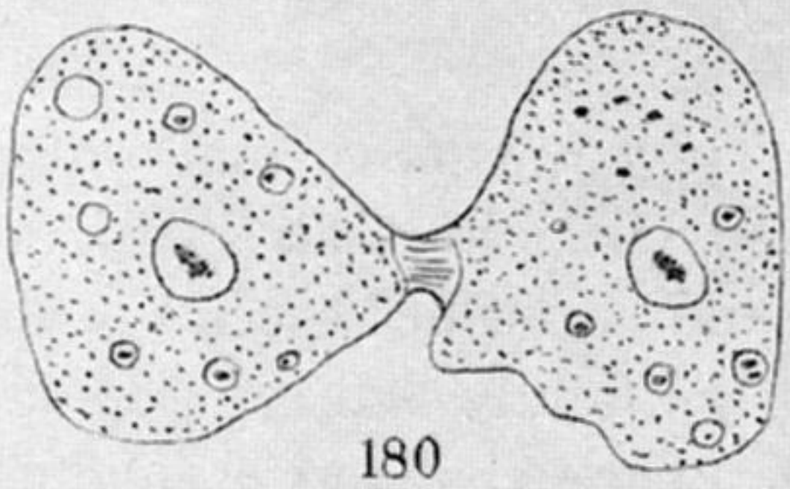
FIGURES 164 to 179

Hartmannella glebae. FIGURES 164 to 179. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 164. Ordinary individual and the structure of two resting nuclei.

FIGURES 165 to 179. Stages in the division.

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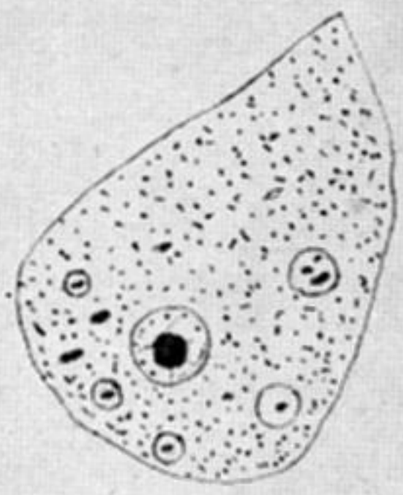
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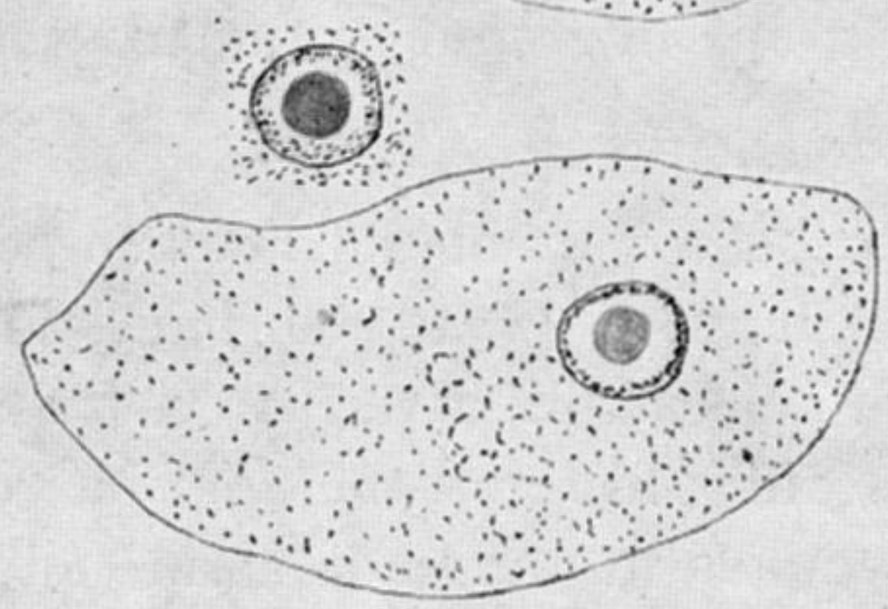
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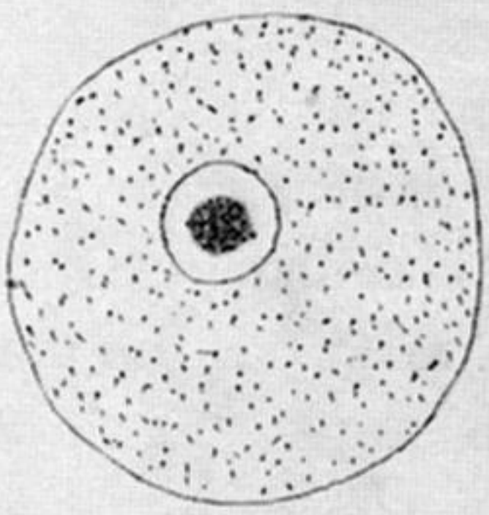
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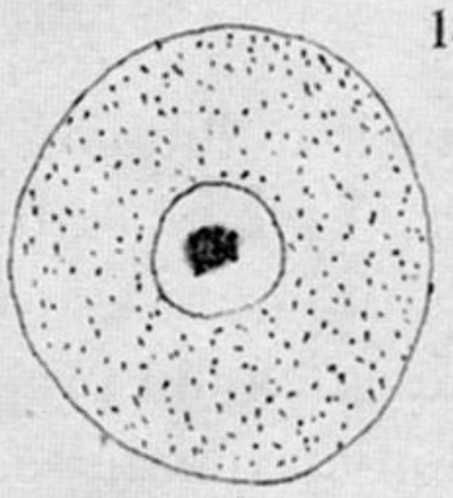
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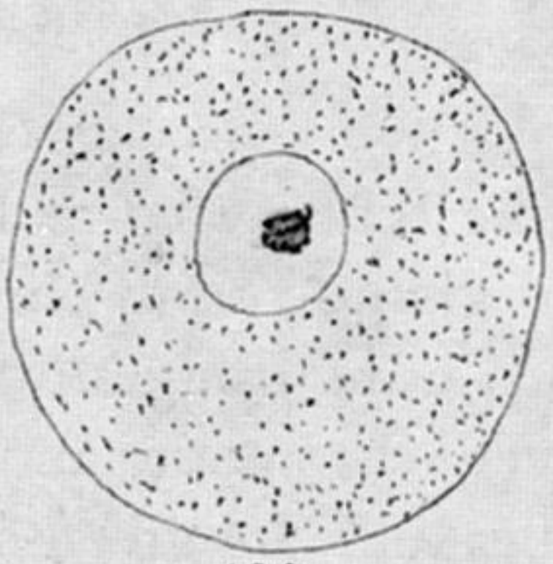
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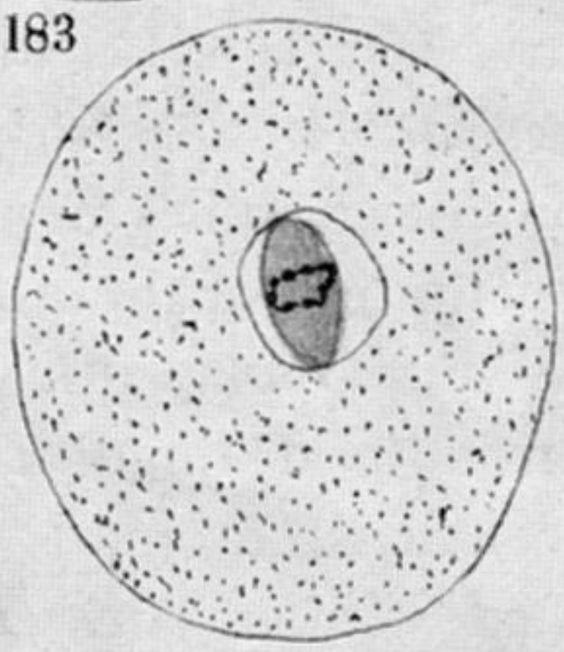
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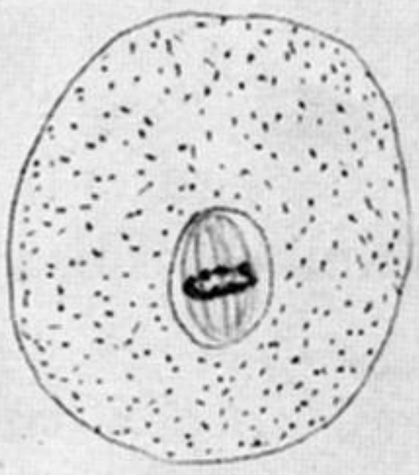
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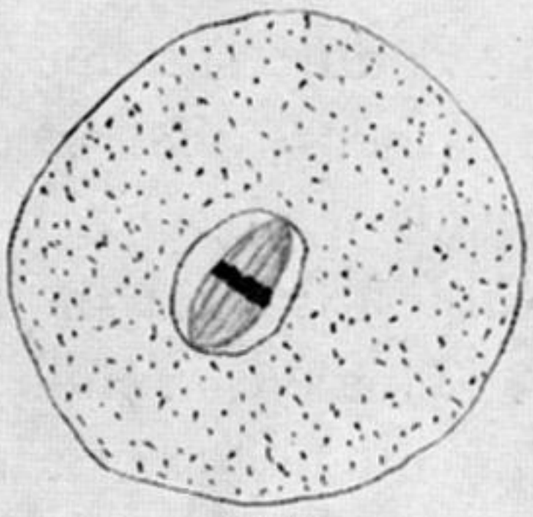
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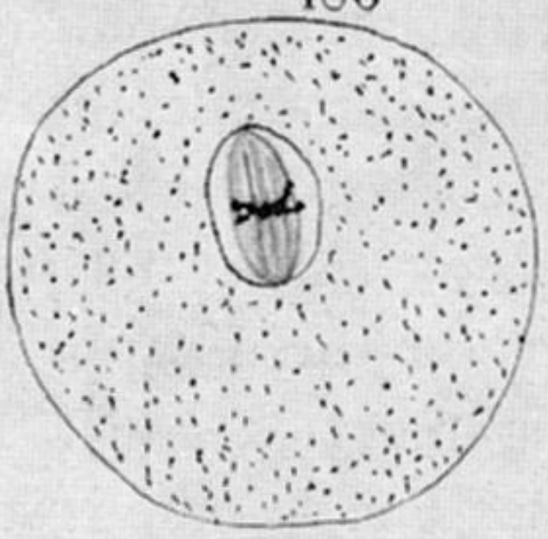
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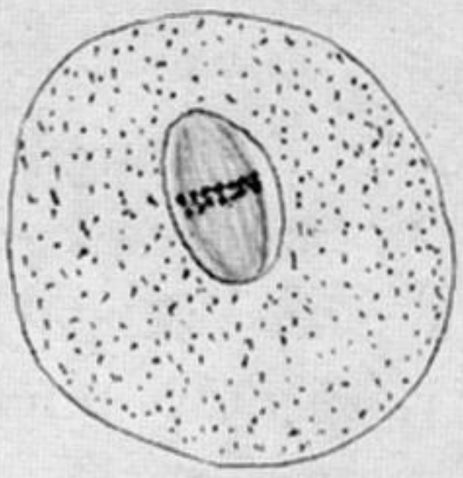
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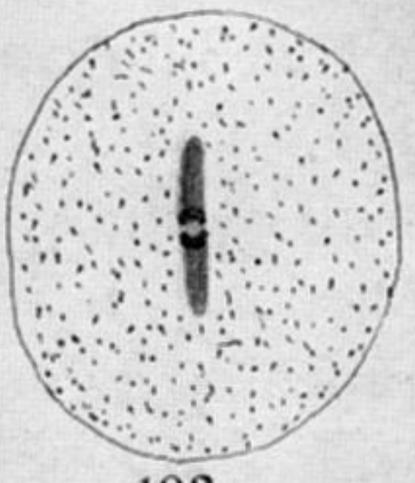
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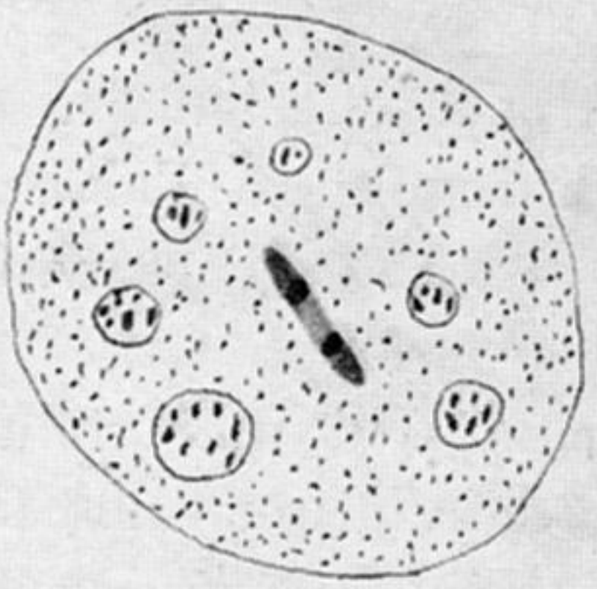
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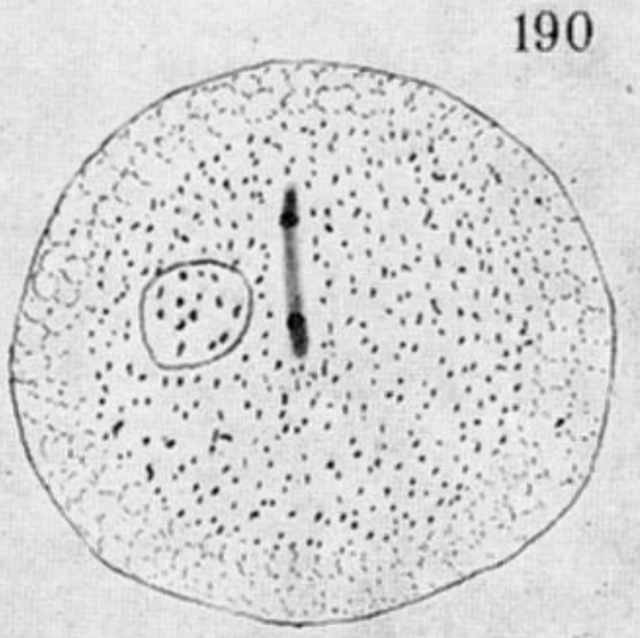
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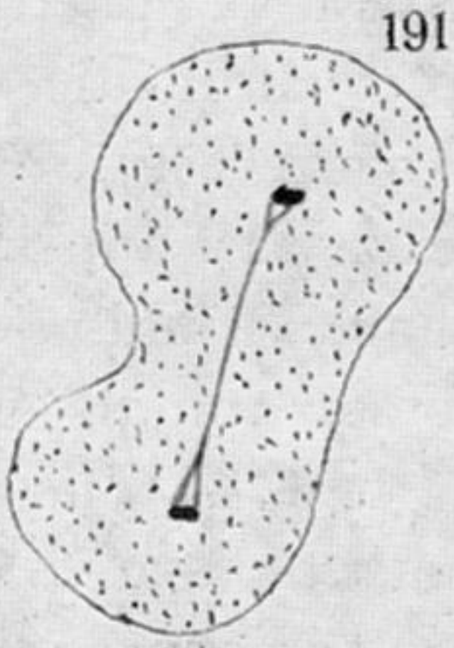
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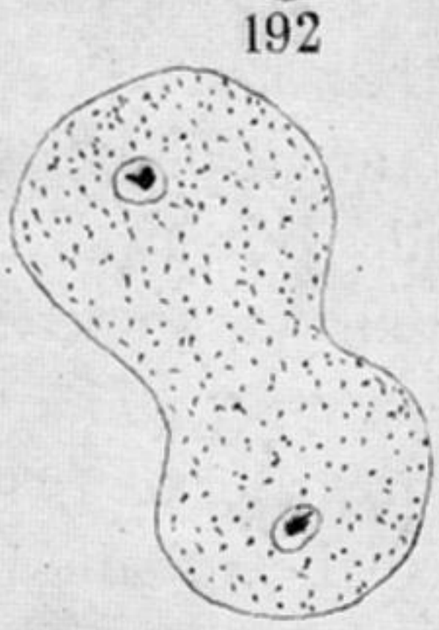
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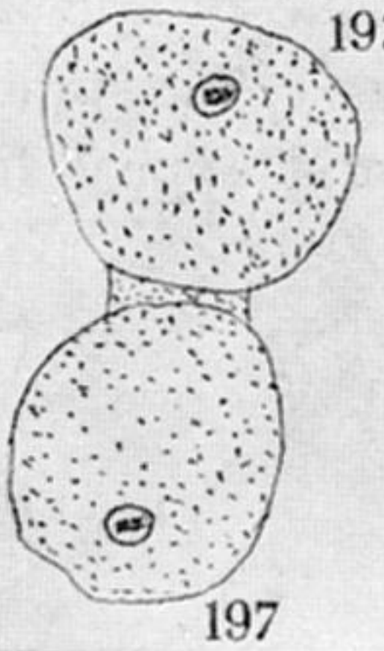
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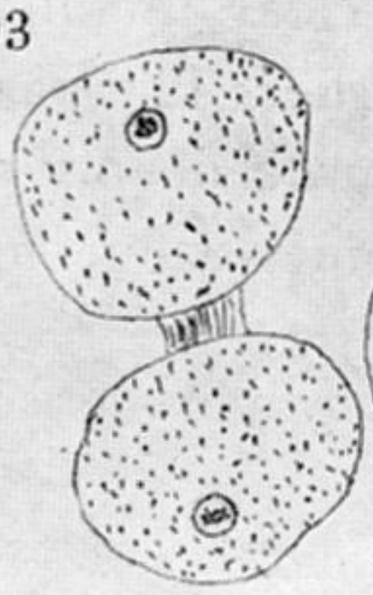
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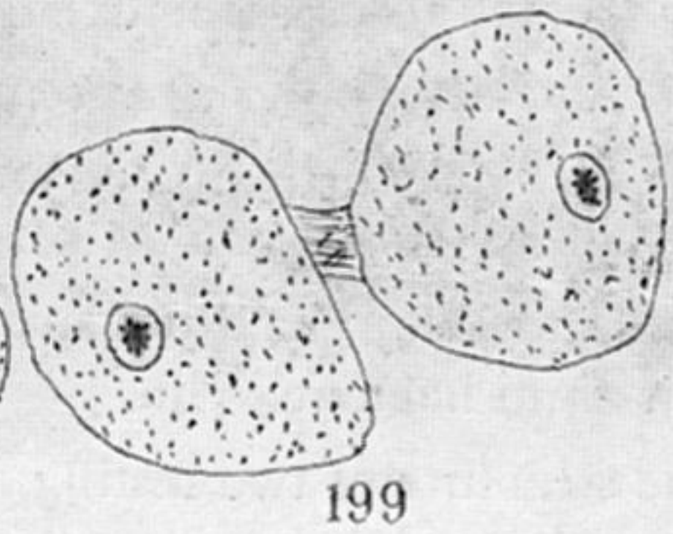
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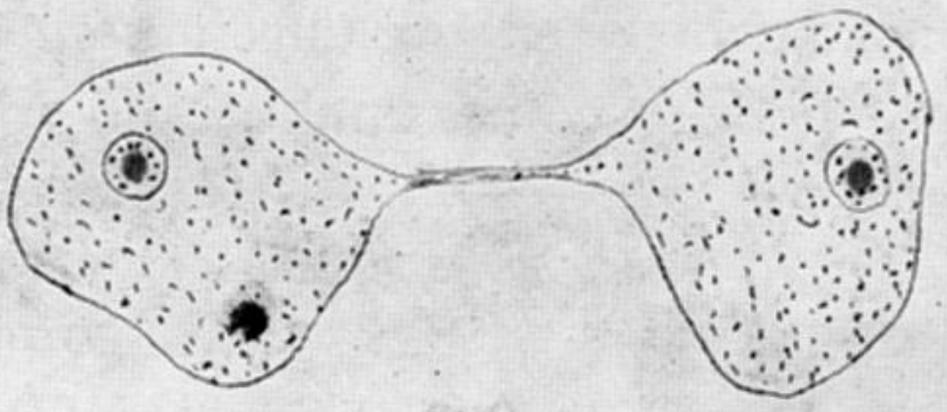

200

Figures 180 to 200

Hartmannella glebae

Figures 180 and 181. Stages in division.
Figure 182. An amoeba which has just divided.
} Fixed in Carnoy and stained with iron-alum haematoxylin

Figures 183 to 200. Fixed in Carnoy and stained with Feulgen reaction and light green.

Figure 183. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

Figures 184 to 200. Successive stages in division. Feulgen-positive chromatic granules give rise to chromosomes and the nucleolus disappears.

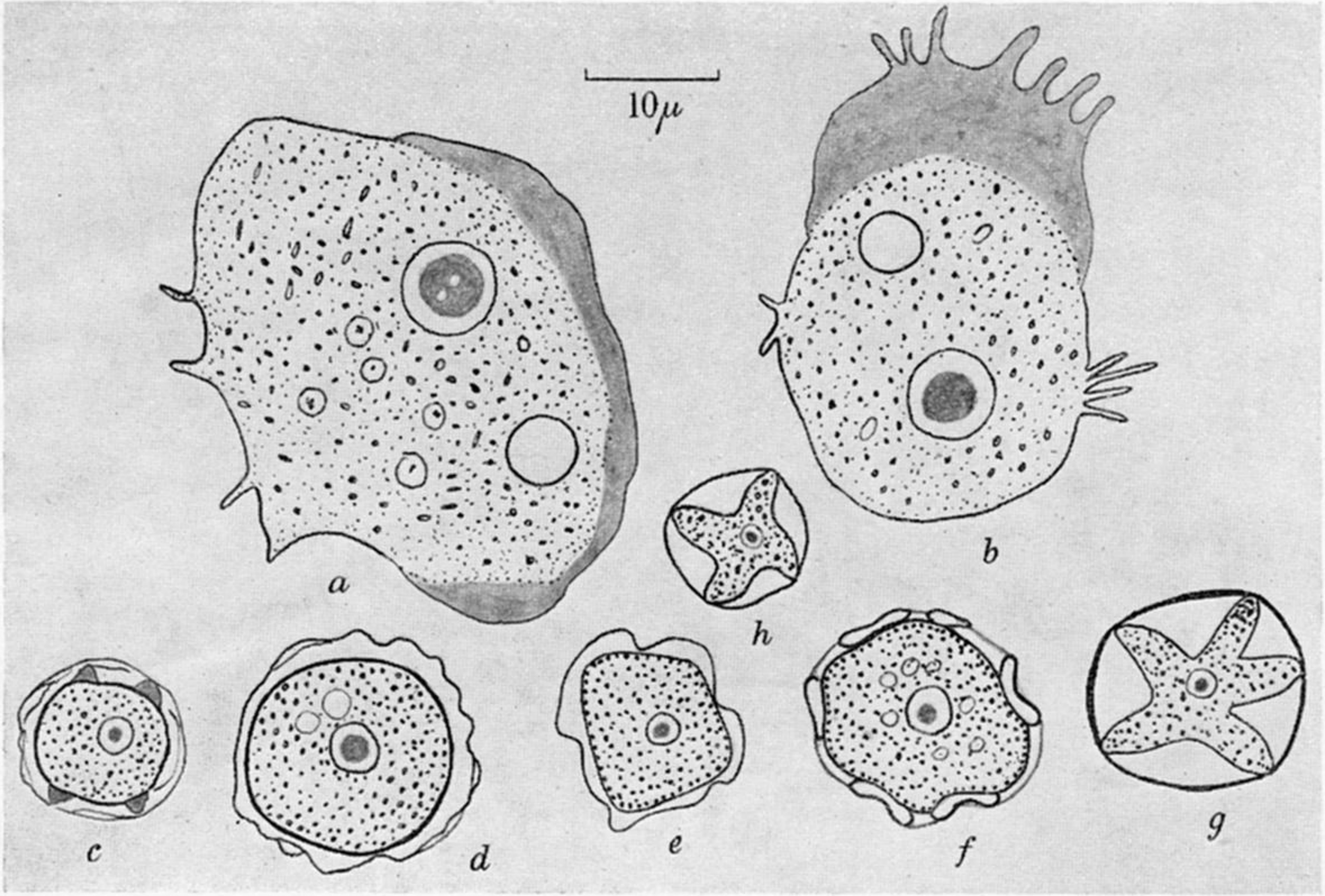
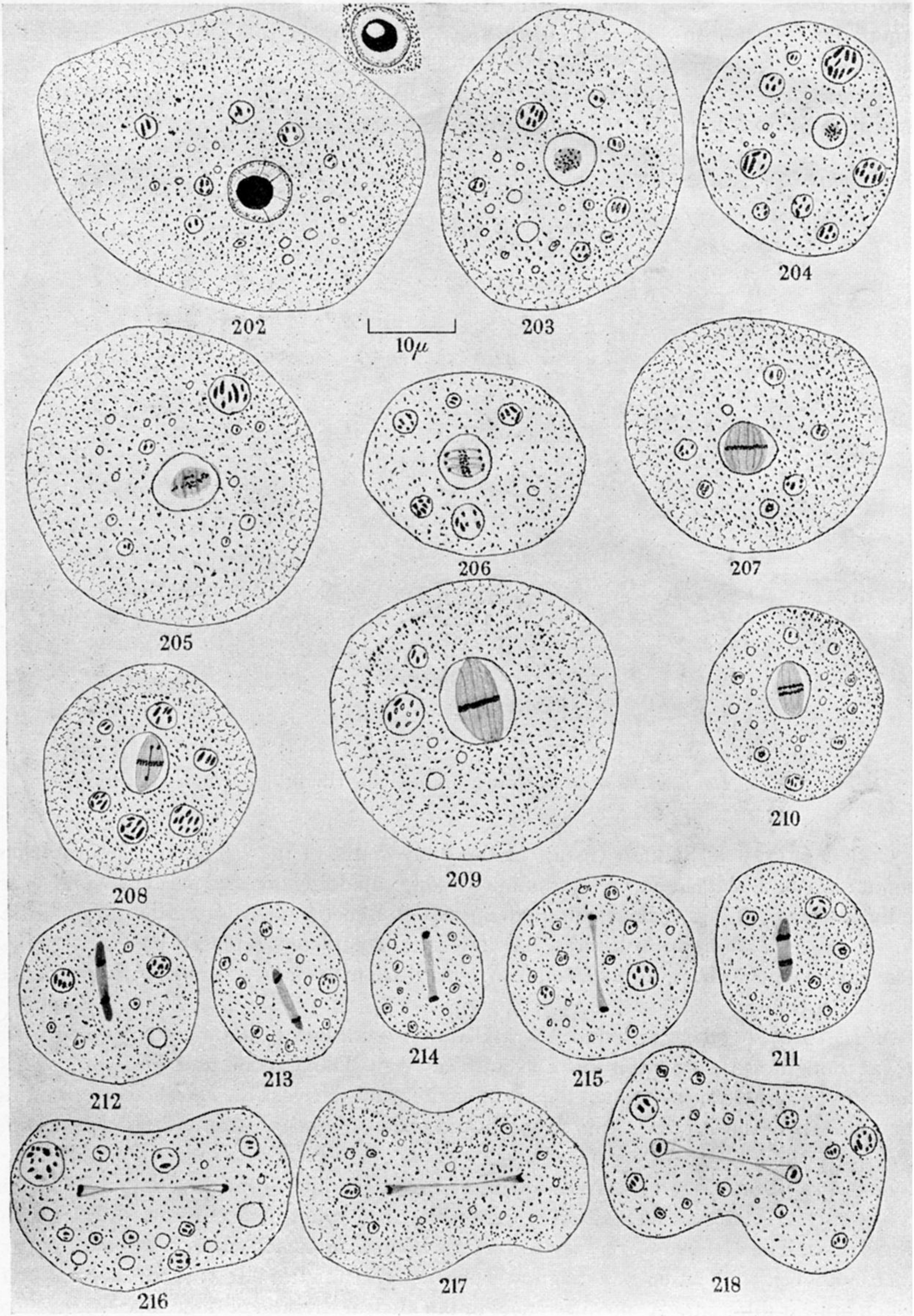


FIGURE 201. *Hartmannella rhysodes*, drawn in the living condition. *a*, *b*, trophic forms; *c*, *d*, *e*, *f*, *g*, *h*, cysts.

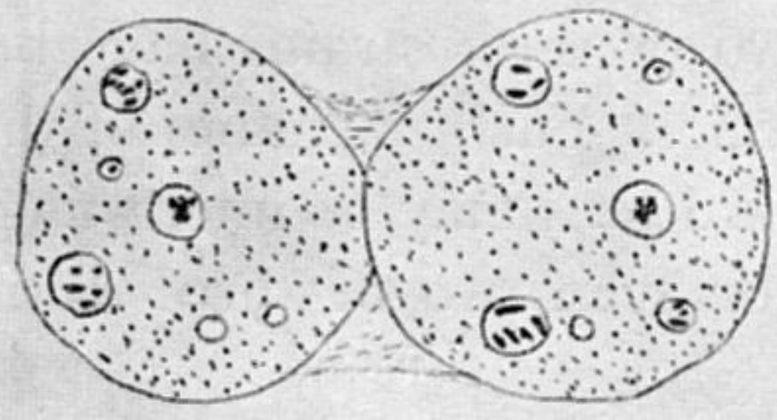


FIGURES 202 to 218

Hartmannella rhysodes. FIGURES 202 to 218. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 202. Ordinary individual and the structure of two resting nuclei.

FIGURES 203 to 218. Successive stages in division.



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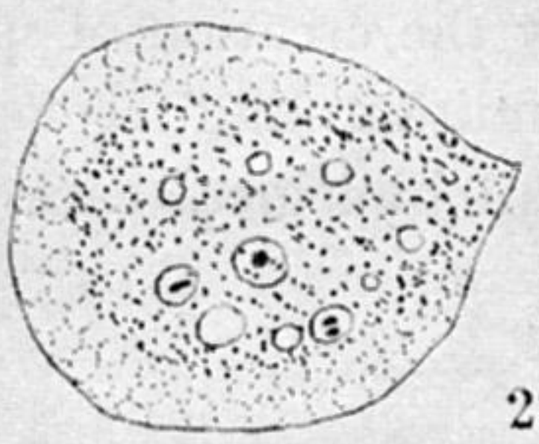

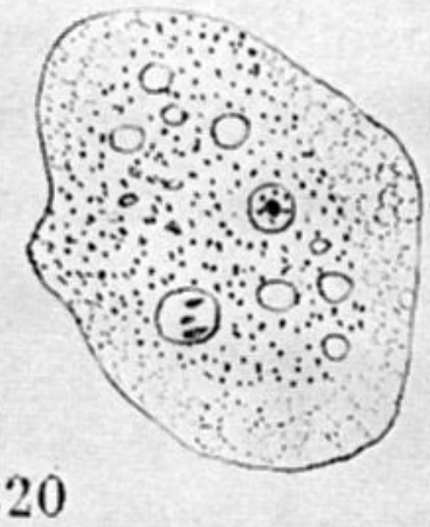

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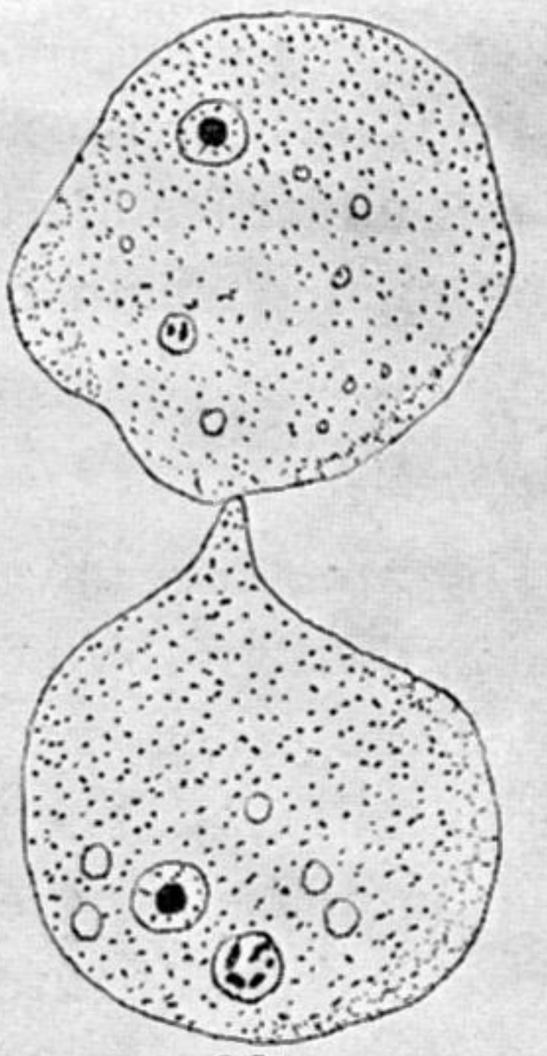

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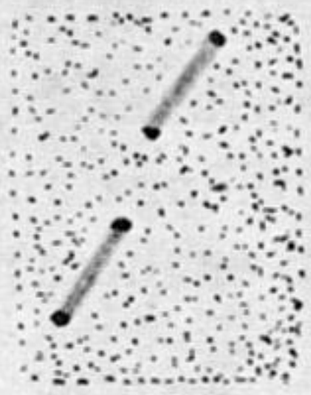

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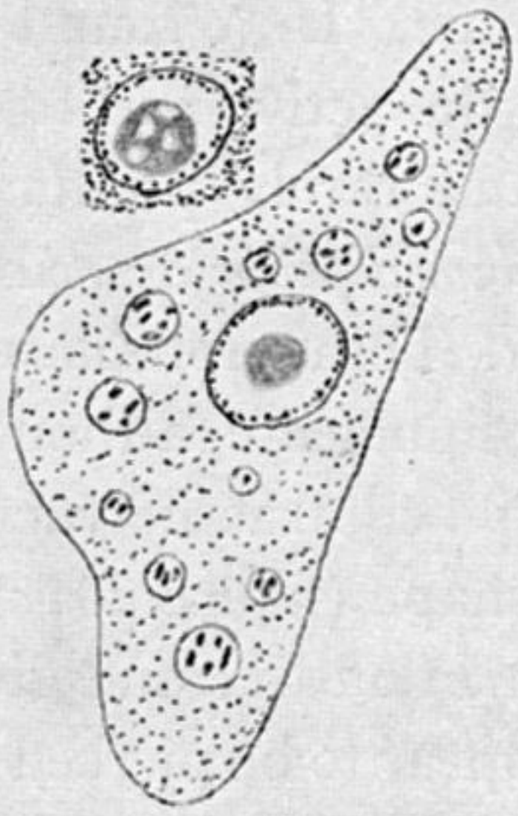

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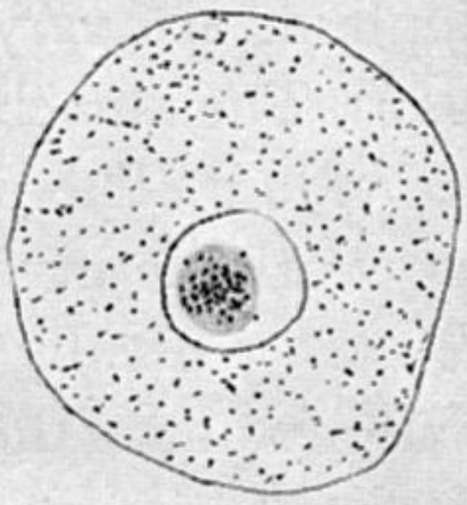

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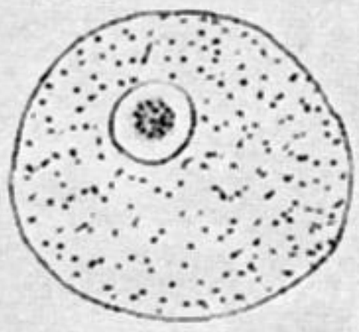

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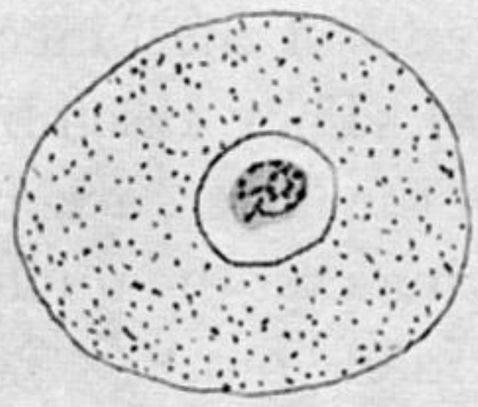

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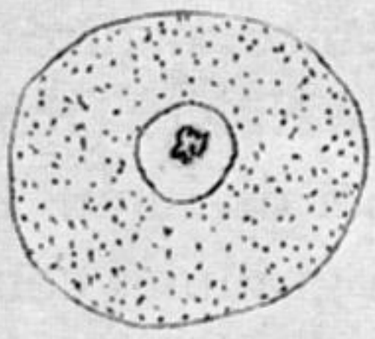

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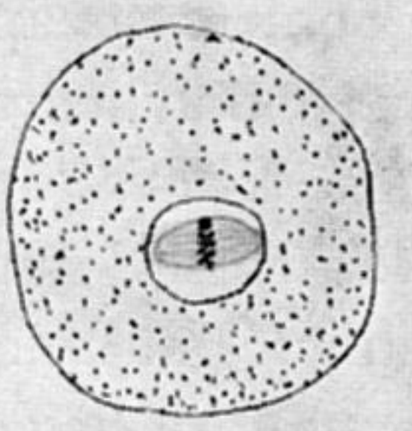

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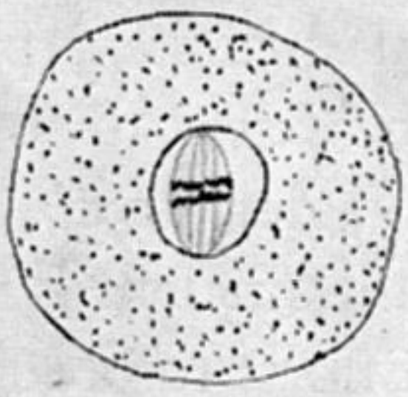

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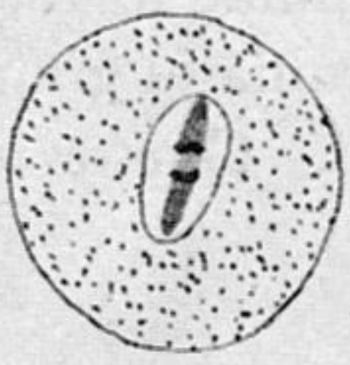

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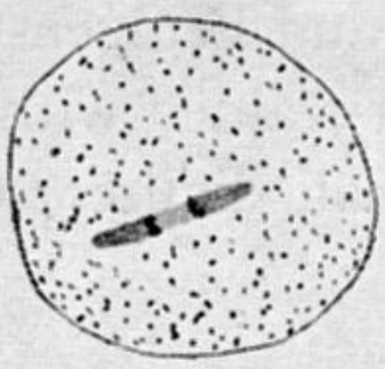

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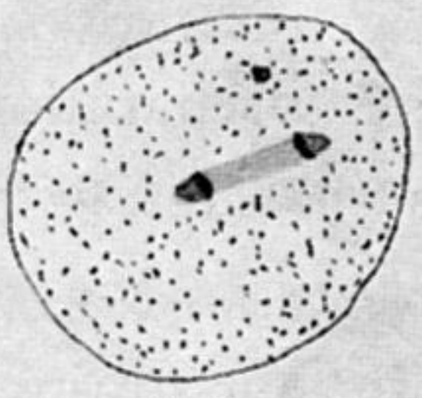

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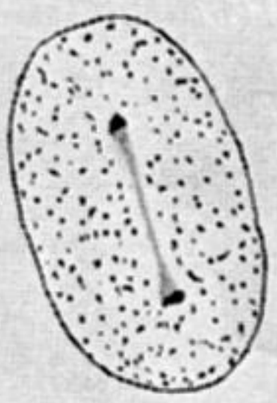

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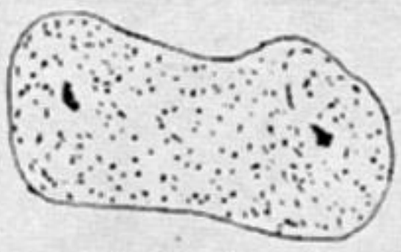

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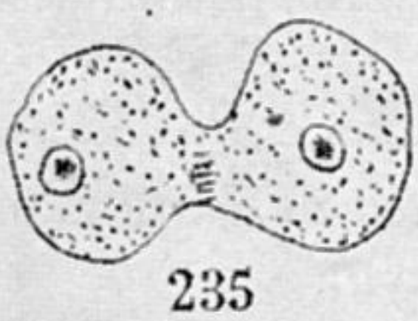

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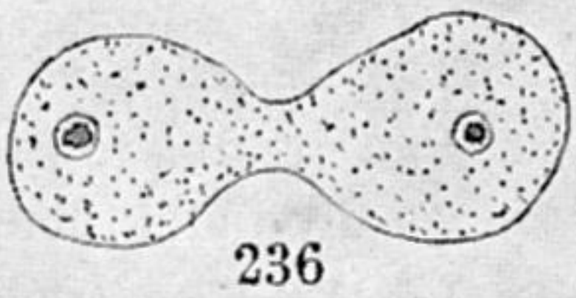

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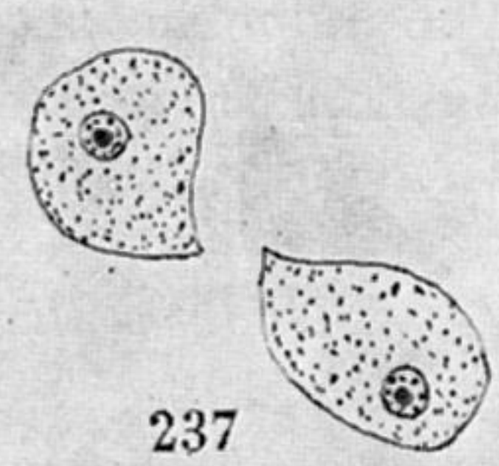

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Figures 219 to 237

Hartmannella rhysodes

Figures 219 to 221. Stages in division.

Figure 222. A stage in the nuclear division of an amoeba having two nuclei.

} Fixed in Carnoy and stained with iron-alum haematoxylin.

Figures 223–237. Fixed in Carnoy and stained with Feulgen reaction and light green.

Figure 223. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

Figures 224 to 237. Successive stages in division. Feulgen-positive chromatic granules give rise to chromosomes and the nucleolus disappears.

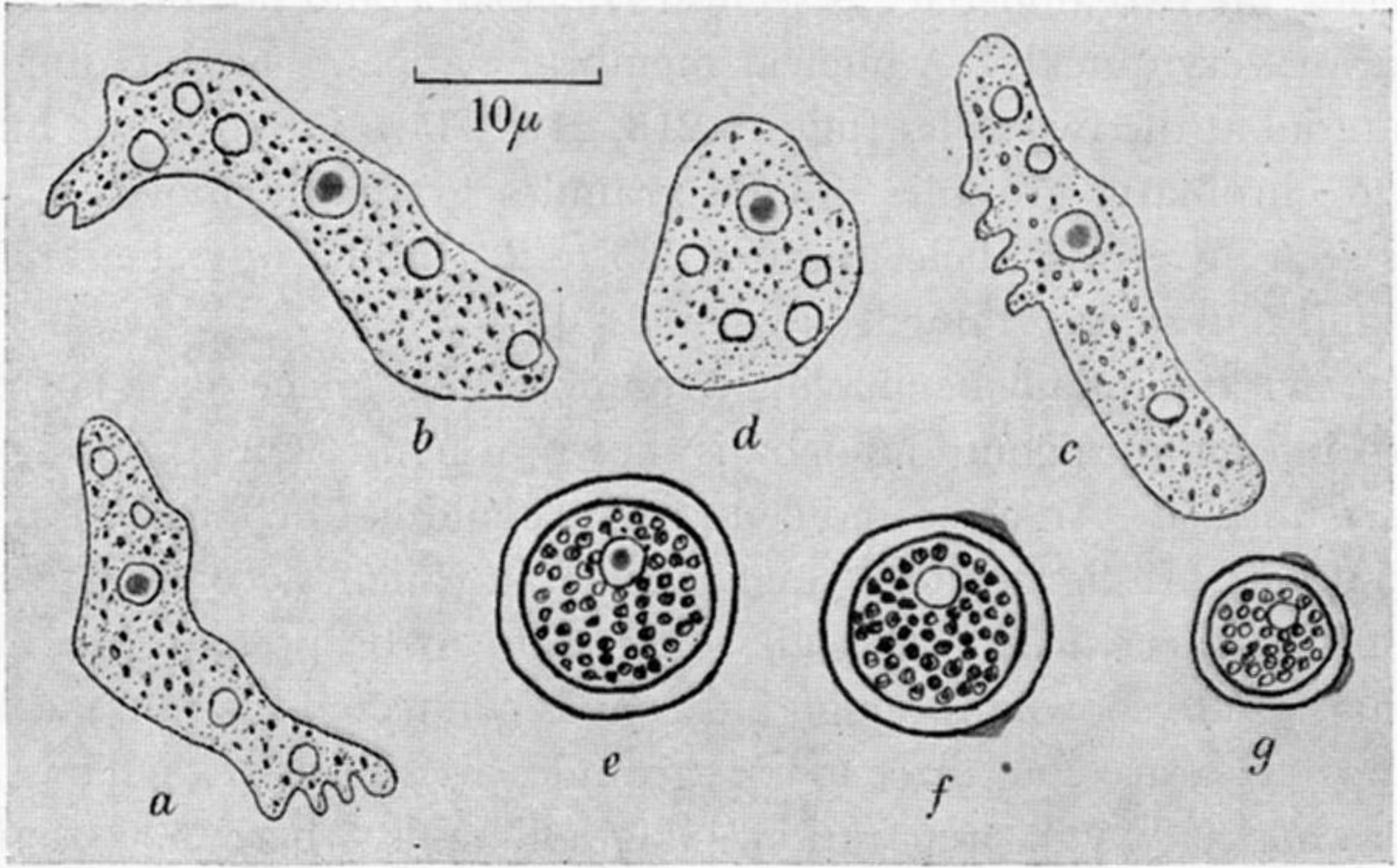
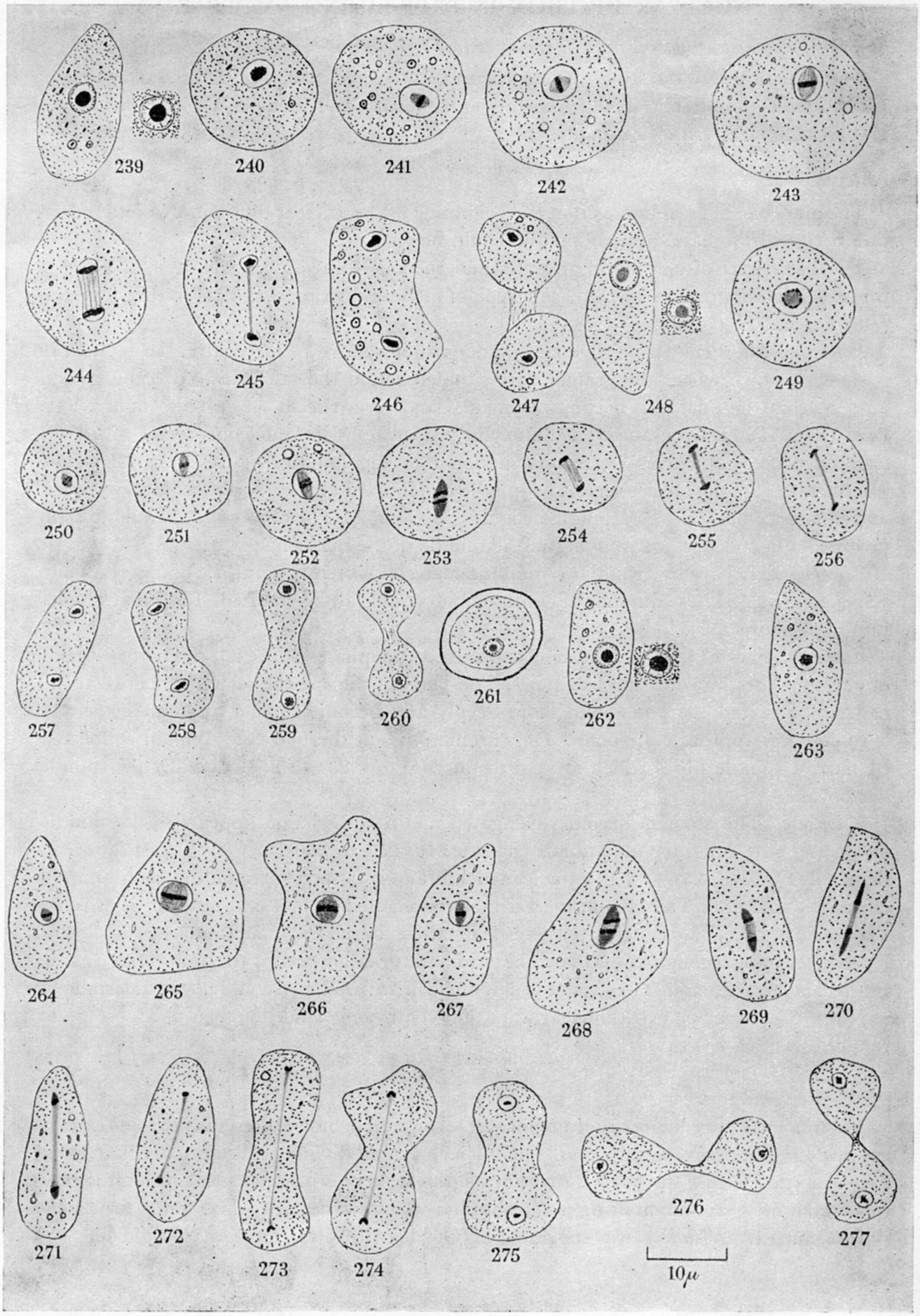


FIGURE 238. *Hartmannella leptocnemus*, drawn in the living condition. *a*, *b*, *c*, *d*, trophic forms; *e*, *f*, *g*, cysts.



FIGURES 239 to 277

Hartmannella leptocnemus

FIGURE 239. Ordinary individual and the structure of two resting nuclei.

FIGURES 240 to 247. Stages in division.

} Fixed in Carnoy and stained with iron-alum haematoxylin.

FIGURES 248 to 261. Fixed in Carnoy and stained with Feulgen reaction and light green.

FIGURE 248. Ordinary individual and two resting nuclei showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules.

FIGURES 249 to 260. Successive stages in division. Feulgen-positive chromatic granules give rise to chromosomes and the nucleolus disappears.

FIGURE 261. A cyst showing Feulgen-negative nucleolus and Feulgen-positive chromatic granules in the nucleus.

Hartmannella agricola. FIGURES 262 to 277. Fixed in Carnoy and stained with iron-alum haematoxylin

FIGURE 262. Ordinary individual and the structure of two resting nuclei.

FIGURES 263 to 277. Successive stages in division.

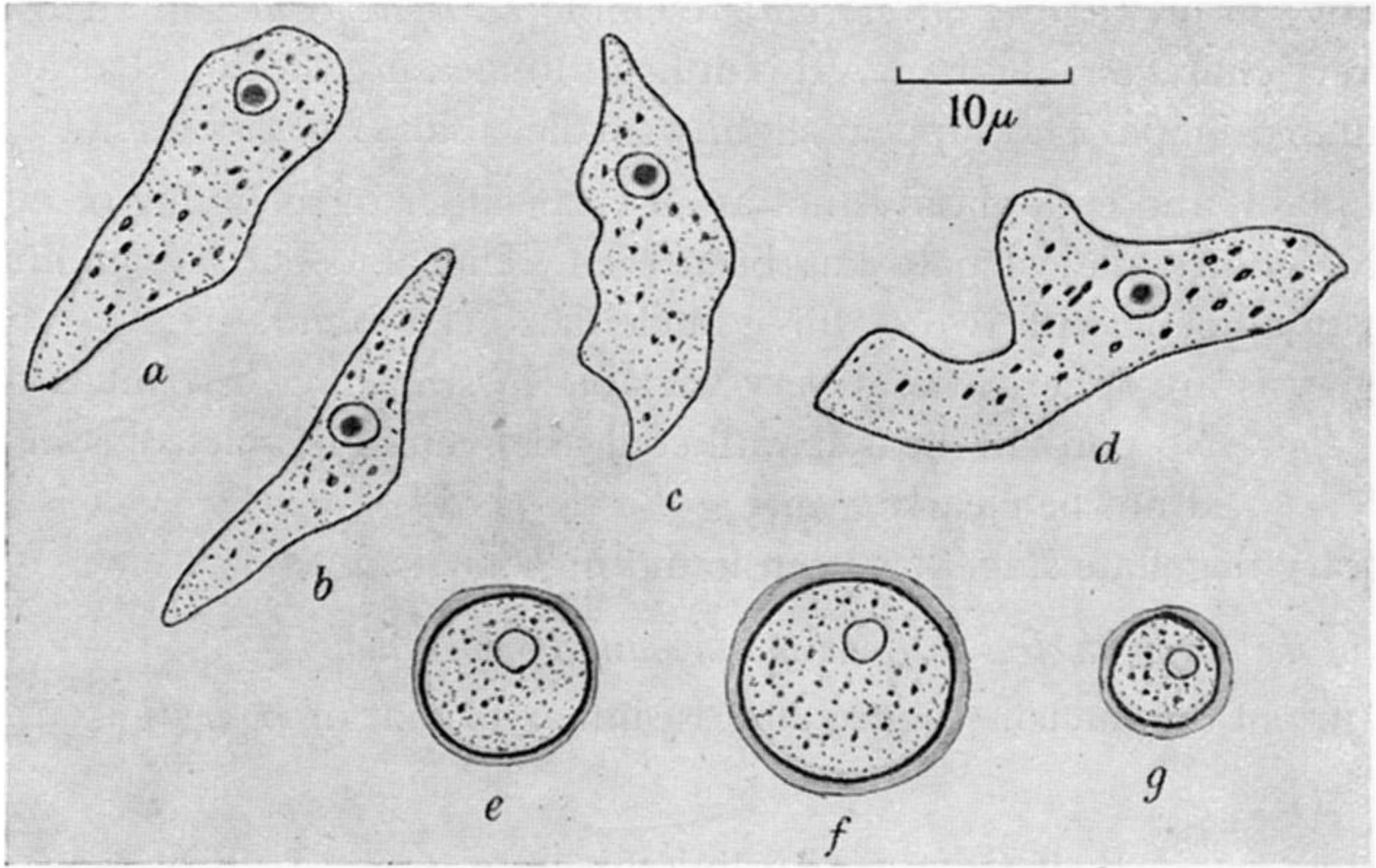


FIGURE 278. *Hartmannella agricola*, drawn in the living condition. *a*, *b*, *c*, *d*, trophic forms; *e*, *f*, *g*, cysts.